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REVIEW ARTICLE

Cancer epigenetics in clinical practice

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Abstract

Cancer development is driven by the accumulation of alterations affecting the structure and function of the genome. Whereas genetic changes disrupt the DNA sequence, epigenetic alterations contribute to the acquisition of hallmark tumor capabilities by regulating gene expression programs that promote tumorigenesis. Shifts in DNA methylation and histone mark patterns, the two main epigenetic modifications, orchestrate tumor progression and metastasis. These cancer-specific events have been exploited as useful tools for diagnosis, monitoring, and treatment choice to aid clinical decision making. Moreover, the reversibility of epigenetic modifications, in contrast to the irreversibility of genetic changes, has made the epigenetic machinery an attractive target for drug development. This review summarizes the most advanced applications of epigenetic biomarkers and epigenetic drugs in the clinical setting, highlighting commercially available DNA methylation-based assays and epigenetic drugs already approved by the US Food and Drug Administration.

KEYWORDS
cancer, epigenetics, DNA methylation, epigenetic biomarkers, epigenetic drugs

INTRODUCTION

Epigenetic modifications are defined as heritable changes in gene activity that do not involve changes in the underlying DNA sequence.[1] Fine tuning of gene expression programs by epigenetic factors is a master molecular mechanism controlling crucial biologic processes, such as cell differentiation and embryogenesis, and there is strong evidence of the relevance of epigenetic reprogramming as a driving force in the dynamic transcriptomic heterogeneity in cancer.[2] The most widely studied epigenetic modification in humans is DNA methylation. Ever since aberrant DNA methylation was first identified in primary human tumors 4 decades ago,[3] comprehensive studies have strongly demonstrated that shifts in the DNA methylation patterns orchestrate tumor progression and metastasis.[4] DNA methylation is a covalent modification that occurs on cytosine nucleotides, almost exclusively at cytosines followed by guanine (CpG sites). The methylation patterns are precisely regulated by a set of enzymes that introduce the modification through either de novo methylation (DNA methyltransferases [DNMTs] DNMT3A and DNMT3B), removal of the methyl group (ten-eleven translocation enzymes TET1, TET2, and TET3), or the full copying and preservation of the methylation patterns during DNA replication (DNMT1; Figure 1). DNA methylation can also be removed passively through sequential cell divisions in the absence of DNA methylation maintenance. CpG sites are not randomly distributed in the genome; instead, there are CpG-rich zones, known as CpG islands, located mainly at the regulatory regions of more than one half of all human genes.[5] Methylation of CpG islands is an epigenetic mechanism of

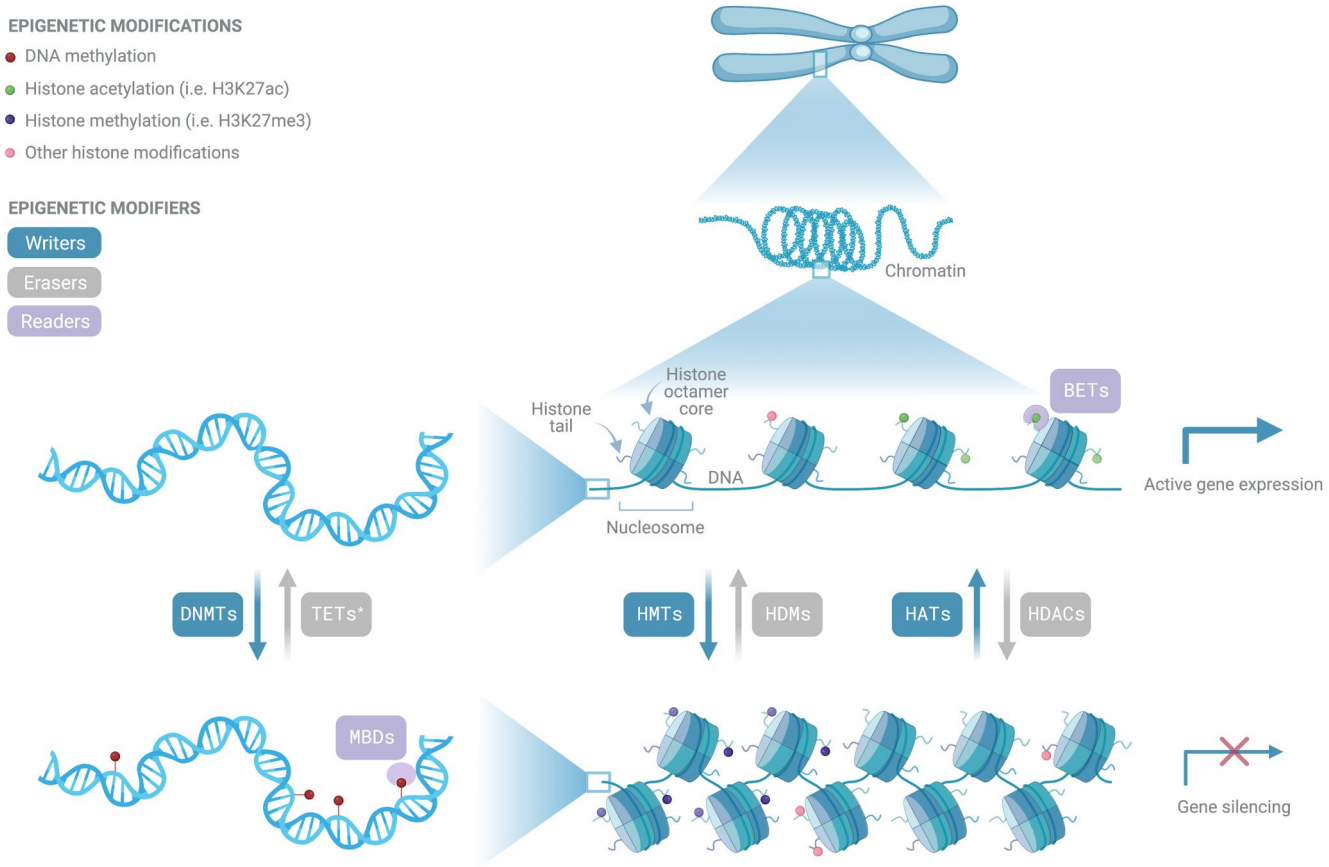

FIGURE 1 Epigenetic machinery shapes chromatin conformation and regulates genome function. DNA is highly condensed and wrapped around a histone octamer core to form a nucleosome, which is the fundamental subunit of chromatin. Epigenetic modifications, including DNA methylation and histone marks, form a complex regulatory network that modulates chromatin structure and genome function. Epigenetic players include enzymes that introduce (writers), recognize (readers), and remove (erasers) epigenetic marks to DNA or histone tails. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) and is removed by ten-eleven translocation enzymes (TETs) or passively through sequential cell divisions (*). Several histone modifications have been described; acetylation and methylation are depicted here because they are the most widely studied histone marks. Histone methylation status is determined by the opposing actions of histone methyltransferases (HMTs) and histone demethylases (HDMs). The same interplay occurs between histone acetyltransferases (HATs) and histone deacetylases (HDACs), which add or remove acetyl groups to lysine residues in the histone tails. This epigenetic code is interpreted by reader or effector proteins that specifically bind to a certain type of modification as methyl-CpG–binding domain proteins (MBDs), which bind to methylated DNA, or as bromodomain and extraterminal domain proteins (BETs), which recognize acetylated lysines. By remodeling chromatin conformation, epigenetic modifications trigger transcriptional silencing or activation via recruitment of other proteins (figure created with BioRender.com). ac indicates acetylation; H3, histone 3; K, lysine; me3, trimethylation.

transcriptional repression. It is a rare event in normal cells, restricted to X chromosome-silencing imprinted genes, germline-specific (ovum, spermatozoid) genes, and some tissue-specific genes.[6] However, promoter-associated CpG island hypermethylation, which was first described as a silencing mechanism of tumor suppressor genes, is a common hallmark in cancer cells.[7,8] In contrast, gene body (introns and exons) methylation is common in active genes in physiologic settings,[9] but a massive global loss of DNA methylation occurs in cancer, mainly at repetitive sequences, that promotes chromosomal instability and reactivation of endoparasitic sequences (a type of transposable element that is repeated at multiple genetic loci).[10,11]

Along with altered DNA methylation profiles, there is also an aberrant landscape of histone modifications in cancer.[12] Together, these epigenetic changes profoundly disturb the transcriptome and consequently disrupt cellular homeostasis. A core of eight histone proteins provides a scaffold to wrap and condense DNA in the nucleus, forming a nucleosome, which is the basic repeating subunit of chromatin (Figure 1). Histone posttranslational modifications (PTMs) are a versatile set of epigenetic marks that, together with DNA methylation, can modulate chromatin conformation and accessibility of transcription factors, co-activators, and co-repressors. The PTMs occurring at the histone tails include acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, and ADP ribosylation, among others. The cross-talk among the different marks configures the so-called *histone code*, which dictates the chromatin structure in which DNA is packaged, and can orchestrate the ordered recruitment of enzyme complexes to wrap the DNA. This histone code is *written* by histone-modifying enzymes that catalyze the introduction of chemical modifications in a residue-specific manner (e.g., histone lysine methyltransferases or

histone lysine acetyltransferases) and is *erased* by enzymes that remove the marks (e.g., histone lysine demethylases or histone lysine deacetylases). This code is interpreted by *reader* or *effector* proteins that specifically bind to a certain type of histone modification or a combination of histone modifications and translate the histone code into a meaningful biologic outcome, whether it is transcriptional activation, or silencing, or other cellular responses (Figure 1). In addition to this recruitment mechanism, histone marks can modulate the chromatin conformation per se based on steric or charge interactions. For instance, neutralization of the positive charges of histones by the acetylation of lysines weakens the histone tail—DNA interactions that lead to chromatin decompaction, which facilitates DNA accessibility.[13,14] Miswriting, misinterpretation, and mis-erasing of histone modifications are linked to oncogenesis. Disturbance of the histone code leads to deregulated gene expression and perturbation of cellular identity; therefore, it is a major contributor to cancer initiation, progression, and metastasis.[14]

In recent years, the emergence of high-throughput technologies has accelerated and expanded our knowledge about the epigenetic mechanisms governing tumorigenesis, revealing a plethora of cancer-specific epigenetic marks or signatures of potential use as biomarkers to define diagnosis, prognosis, or response to therapies. Moreover, the reversibility of epigenetic changes, in contrast to the irreversibility of genetic changes, makes the epigenetic machinery an attractive target for drug development, which is an active field of research. Several companies exclusively dedicated to the epigenetic market have been launched during the last years, and the fastest growing are located in Asia. In this review, we provide an overview of the epigenetic contributions to clinical oncology, through epigenetic biomarkers and epigenetic drugs, focusing on the most advanced applications in the clinical setting. Commercially available DNA methylation-based assays of clinical utility, as well as the epigenetic drugs already approved by the US Food and Drug Administration (FDA), are highlighted.

EPIGENETIC BIOMARKER LANDSCAPE IN CANCER

Today, the clinical implementation of genomic biomarkers predictive of a response to matched targeted therapies is a reality, and efforts are being made to develop policies for establishing personalized pharmacogenetic prescriptions in health care systems to broaden the access to biomarker testing. One example of success in this respect is the use of activating *EGFR* mutations as biomarkers for treatment with EGFR inhibitors, resulting in a substantial improvement in survival over time in patients with nonsmall cell lung cancer (NSCLC) that can be ascribed to the timing of approval of EGFR-targeted therapy.[15] In another example, genetic alterations of epigenetic players, such as enhancer of zeste homolog 2 (*EZH2*) or, indirectly, isocitrate dehydrogenase 1 (*IDH1*) and *IDH2* mutations, have been targets for drug development and are currently used as biomarkers for treatment, as explained below (see Epigenetic drugs).

As with genetic biomarkers, there is increasing evidence that epigenetic biomarkers can aid traditional pathology to improve clinical management and patient outcomes. Epigenetic characterization of human tumors has revealed characteristic patterns that can be useful for precise diagnosis or even for defining novel tumor subtypes, recurrence detection, residual disease monitoring, or to guide treatment decision making (Figure 2). The sections below describe the current epigenetic biomarker landscape in cancer, focusing on the most advanced examples with clear utility in clinical oncology. Considering that the vast majority are DNA methylation biomarkers, a brief introduction about the methods available for analyzing this epigenetic modification as well as sample requirements is included, emphasizing the use of liquid biopsy as a suitable noninvasive approach.

Methods for analyzing DNA methylation

Aberrant DNA methylation in cancer was first detected by Southern hybridization using restriction endonucleases that discriminate between methylated and unmethylated CG sequences, such as HpaII, HhaI, or NotI.[3,16,17] A crucial advance in the analysis of DNA methylation resulted from the demonstration that treatment of DNA with sodium bisulfite deaminates the unmethylated cytosines, converting them to uracil, while leaving methylated cytosines intact.[18] Taking advantage of the sequence differences resulting from bisulfite modification, the use of bisulfite-treated DNA created myriad possibilities to explore DNA methylation. First, assays were developed to perform locus-specific analysis of candidate genes. Using primers designed to distinguish methylated from unmethylated CpGs in bisulfite-modified DNA, methylation-specific polymerase chain reaction (PCR)[19] was a pivotal method to establish the relevance of promoter-associated CpG island hypermethylation in cancer and to identify potential biomarkers of clinical utility.[20]

More recently, the emergence of high-throughput strategies has enabled the genome-wide mapping of methylated cytosines in bisulfite-treated DNA. The complete landscape of DNA methylation at single-nucleotide resolution can be obtained by whole-genome bisulfite sequencing,[21] although high sequencing costs and the need for specialized computational analysis have limited the application in clinical practice. To decrease the cost of whole-genome bisulfite sequencing, reduced-representation bisulfite sequencing[22] technology was developed to sequence a smaller representative sample of the whole genome. By using a methylation-insensitive CpG restriction endonuclease (typically MspI) to generate CpG-enriched fragments at the ends, this approach captures 85% of CpG islands.[23] Another alternative to decrease costs by sequencing a limited part of the DNA methylome is to enrich the DNA fragments that are putatively methylated. This methodology is based on using antibodies directly against methylated DNA (MeDIP)[24] or against methyl-CpG–binding domain proteins (MBDs), which have a high affinity for binding methylated cytosines.[25] The immunoprecipitated DNA is then sequenced (MeDIP-seq or MBD-seq) to profile DNA methylation.[26] Together, these technologies have generated important knowledge about DNA

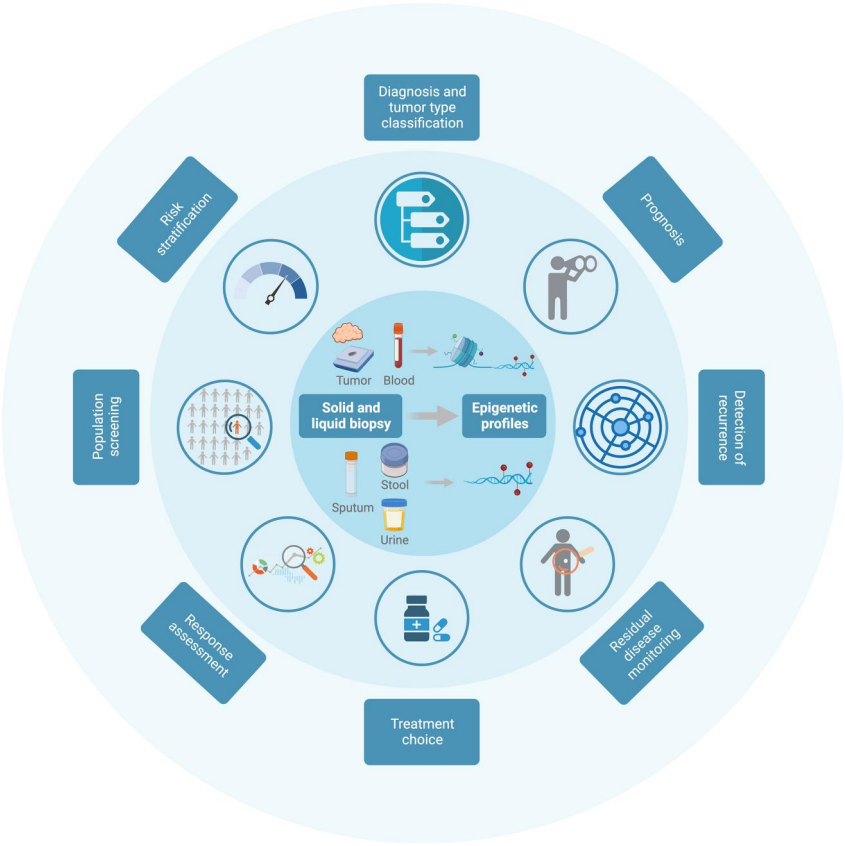

FIGURE 2 Clinical applications of epigenetic biomarkers. Cancer specificity of the epigenetic profiles generated not only from tumor tissues (solid biopsies) but also from body fluids, such as blood, urine, stool, or sputum (liquid biopsies), make them an invaluable source of biomarkers to aid disease diagnosis, monitoring, and treatment choice. Stability of DNA methylation in circulating tumor DNA has promoted the development of DNA methylation-based assays as an ideal noninvasive strategy for patient surveillance (figure created with BioRender.com).

methylation in physiologic and pathologic settings, including the epigenetic mechanisms governing tumorigenesis.

However, the most comprehensive sets of DNA methylation profiles in human cancer have been generated using bisulfite conversion-dependent methylation arrays, which are cost-effective platforms for genome-wide methylation analysis. The use of the Infinium HumanMethylation450 BeadChip (450K) arrays (Illumina) has been broadly extended, potentiated by its use as the platform of choice for The Cancer Genome Atlas (TCGA) studies[27] and its versatility in determining DNA methylation patterns from formalin-fixed, paraffin-embedded (FFPE) samples.[28] The 450K array interrogates the methylation status of approximately 450,000 CpGs located not only at CpG islands, shores, and shelfs surrounding the transcription start sites for coding genes but also at gene bodies and 3′-untranslated regions, in addition to intergenic regions derived from genome-wide association studies.[29] The 450K DNA methylation profiles of 11,315 TCGA samples across 33 different tumor types available at the Genomic Data Commons Data Portal (https://portal.gdc.cancer.gov/) are an invaluable resource for cancer research. Moreover, increasing data from the most recent Infinium array version, the Human MethylationEPIC BeadChip, which interrogates almost a million CpGs, incorporating CpG sites located in enhancer regions identified by the ENCODE and FANTOM5 projects,[30] is providing a more thorough epigenomic characterization of human tumors. Several DNA methylation array-based classifiers and epigenetic signatures of clinical relevance have been developed using comprehensive machine-learning approaches. Examples include: DNA methylation-based classifiers for central nervous system (CNS) tumors,[31] sarcomas,[32–34] and cutaneous melanoma[35]; an epigenetic-based tumor type classifier to predict tumor origin in cancer of unknown primary (CUP)[36]; and a predictor of response to immunotherapy indicating which patients with NSCLC are most likely to benefit from anti–PD-1 agents.[37]

Although bisulfite treatment is the gold standard method for mapping DNA methylation, third-generation sequencing approaches, including nanopore sequencing by Oxford Nanopore Technologies,[38] offer new opportunities for the direct detection of DNA methylation. Nanopore sequencing techniques detect DNA modifications through differences in the electric current intensity produced by nanopore reads of an unmodified and a modified base.[39]

Knowledge generated from all of these genome-wide technologies has been crucial to the expansion of the repertoire of epigenetic biomarkers of clinical utility. Once the CpGs of interest have been identified, target-specific approaches to assess the candidate

biomarkers facilitate the translation to clinical practice. Among the more frequently used technologies are PCR-based assays using bisulfite-treated DNA, including allele-specific quantitative PCR (qPCR); digital-droplet PCR (ddPCR); the highly sensitive MethyLight assay (Epigenomics, Inc.), which incorporates fluorescence-based real-time PCR (TaqMan) technology[40]; and the enhanced MethyLight ddPCR version, which detects infrequently methylated alleles.[41] There are also target-sequencing methods like pyrosequencing,[42] in which the detection system is based on the pyrophosphate released when a nucleotide is introduced in the DNA strand.

Source material for DNA methylation analysis and liquid biopsy

Stability of DNA methylation is a key feature for the clinical utility of this epigenetic modification because it is not affected by sample processing or storage conditions. DNA methylation can be assessed not only in fresh or frozen tissues, but also in FFPE samples,[28] which is the gold standard in clinical practice. Moreover, tumor-derived cell-free DNA (cfDNA) present in body fluids, such as blood, urine, stool, or sputum, is an invaluable source with which to perform noninvasive DNA methylation analyses (Figure 2). The fact that DNA methylation profiles are preserved in blood and nonblood circulating tumor DNA (ctDNA) makes this modification ideal for liquid biopsy. Cost-effective assays can be designed to detect cancer-specific DNA methylation changes for early diagnosis and disease monitoring. However, the tiny amount of ctDNA is a major challenge that must be overcome because the proportion of ctDNA in the background of overall cfDNA is highly variable, ranging from <0.05% to 90%, depending on the tumor volume, localization, vascularization, and tumor type, among other factors.[43] Moreover, the concentration and fraction of ctDNA are highly correlated with cancer stage because ctDNA in plasma can be detected in >80% of patients who have stage IV disease but only in one half of those who have stage I disease.[44] Hence, extremely sensitive assays need to be designed and critical measures taken to maximize assay performance. Specialized collection tubes for cfDNA must be used to avoid the lysis of nucleated cells because the release of large amounts of fragmented DNA may mask the ctDNA signal. Furthermore, the use of preservatives to stabilize urine cfDNA must be considered when using this body fluid as a source of ctDNA because of the high level of activity of DNase I in urine. Moreover, special considerations must be given when designing assays for stool because bacterial DNA can interfere with the analysis.[45]

Although challenging, the noninvasive nature of ctDNA methylation assays and their multiple potential clinical applications have encouraged research in this area, and massive efforts have been made to optimize the methodologies initially developed for analyzing tumor samples. One of the expanding fields is the development of DNA methylation-based assays for population screening, considering that the straightforward use of body fluids, even those obtained by self-collection, can increase participation and compliance rates. Successful examples include Cologuard[46] (Exact Sciences Company) and Epi proColon[47,48] (Epigenomics, Inc.) for colorectal cancer (CRC) screening in stool and blood, respectively, which are described in the section below. Easy access to the molecular information from the tumor in liquid biopsy also enables sequential sampling, facilitating the monitoring of minimal residual disease (MRD) after curative therapies. This feature has been exploited in bladder cancer by using urine as a surrogate sample in assays, such as the Bladder EpiCheck Urine Test (Nucleix Ltd.), as explained below.

DNA methylation-based assays of clinical utility in oncology

To identify the DNA methylation biomarkers in advanced stages of development for clinical oncology purposes, in addition to the literature search of scientific publications, the ClinicalTrials.gov database maintained by the National Library of Medicine, was interrogated on May 17, 2022 using the keywords *cancer AND methylation*. The list of clinical trials (CTs) obtained was first filtered to select *terminated*, *completed*, *active, not recruiting*, *not yet recruiting*, *recruiting*, and *enrolling by invitation* trials. Next, the list was strictly curated to include only CTs directly involving DNA methylation-based strategies. Three categories were established: (1) market: CTs designed to determine the performance (sensitivity and specificity) of DNA methylation-based tests that are currently registered on the market, including CTs that were pivotal in defining biomarker accuracy (Table 1)[46–86]; (2) investigative: CTs designed to assess the performance of previously identified DNA methylation biomarkers (Table 2); and (3) exploratory: CTs used to identify DNA methylation biomarkers (Table 3). Figure 3A summarizes the market, investigative, and exploratory CTs by tumor type.

The massive increase in the number of CTs involving DNA methylation biomarkers in recent years (Figure 3B) demonstrates the growing relevance of epigenetics in clinical oncology. The first two trials were those assessing the performance of *GSTP1* methylation as a marker for the early detection of prostate cancer (PCa; ClinicalTrials.gov identifier NCT00340717), which began in 2003, and the use of *p16/CDKN2A* methylation as a biomarker of the malignant potential of oral epithelial dysplasia, initiated in 2005 (ClinicalTrials.gov identifier NCT00835341).[87] Twenty-seven CTs began in 2021, and 22 more started up during the first one half of 2022 (Figure 3B). Another unequivocal indicator of growing is the number of DNA methylation-based tests launched on the market annually since 2018.

More than 30 DNA methylation-based assays to aid clinical decision making in cancer have reached the market. Detailed descriptions, including information about their clinical use and assay performance (sensitivity and specificity), are provided in Table 1. A significant expansion of the epigenetics portfolio is expected in the coming years because of increasing investment in research and development and the launch of companies exclusively dedicated to providing epigenetic health care solutions.

TABLE 1 Registered DNA methylation-based assays of clinical utility in oncology

TEST	CANCER TYPE	SAMPLE TYPE	USE	MOLECULAR MARKERS	ASSAY TECHNOLOGY	COMPANY (Country)	SENSITIVITY	SPECIFICITY	US APPROVAL	EUROPEAN CERTIFICATION	ASIAN APPROVAL	GUIDELINES	RELATED CLINICAL TRIALS (NCT ID)	RELATED ARTICLES
Cologuard (sDNA-FIT)	Colorectal	Stool	S-ED	*NDRG4* and *BMP3* methylation + 7 *KRAS* mutations	qMSP	Exact Sciences Co. (USA)	92.3% (95% CI: 83.0–97.5) for detecting CRC.	86.6% (95% CI: 85.9–87.2). FPR=10.2-13.4%.	FDA approved (2014)			NCCN CRC screening (v2.2022) ACS CRC screening (2018) USPSTF CRC screening (2021)	NCT01397747 (C); NCT03741166 (C); NCT03728348 (C).	46
Epi proColon/ Epi proColon 2.0 CE	Colorectal (also in CT for HCC screening)	Blood	S-ED	*SEPT9* methylation	MethyLight	Epigenomics, Inc. (Germany)	68% (95% CI: 53–80) for all stage CRC. 64% (95% CI: 48–77) for stage I-III CRC.	80.0% (95% CI: 78–82). NPV=99.7% (95% CI: 99.6–99.8).	FDA approved (2016, Epi proColon)	CE-IVD (2011, Epi proColon 2.0 CE)	NMPA approved (2014, Epi proColon 2.0 CE)	NCCN CRC screening (v2.2022, Epi proColon)	NCT00696345 (C); NCT00855348 (C); NCT01329718 (C); NCT03218423 (R); NCT04027790 (C). (NCT03311152 in HCC, R)	47,48,69
Earlytect Colon Cancer	Colorectal	Stool	S-ED	*SDC2* methylation	LTE-qMSP	Genomictree, Inc. (South Korea)	90.2% (95% CI: 85.8–93.6; n=221/245) overall sensitivity. 89.1% (95% CI: 82.3–93.9; n=114/128) for detecting early stages (0-II).	90.20% (95% CI: 85.8–93.6; n=221/245).		CE-IVD (2017)	MFDS approved (2018)		NCT03146520 (C); NCT04304131 (AnR); NCT05255588 (R).	80,82,83
Colosafe	Colorectal	Stool	S-ED	*SDC2* methylation	qMSP	Creative Biosciences (Guangzhou) Co. Ltd. (China)	84.22% (n=315/374) in stage I-IV. 86.71% (n=137/158) in stage I-II.	97.85% (n=821/839).			NMPA approved (2018)		NCT04030637 (R); NCT04786704 (C); NCT05374369 (C). (NCT04722055 (AnR) Colosafe 2.0).	
ColoClear	Colorectal	Stool	S-ED	*NDRG4* and *BMP3* methylation + *KRAS* mutations	qMSP	New Horizon Health Technology Co. Ltd. (China)	96% for CRC. 64% for advanced adenoma.	87%			NMPA approved (2020)		NCT04287335 (C).	
Colowell	Colorectal	Stool	S-ED	*SDC2* and *SFRP2* methylation		Shanghai Realbio Technology (Co. Ltd. (China)							NCT04515082 (NyR)	
Colvera	Colorectal	Blood	M-R	*BCAT* and *IKZF1* methylation	Multiplexed qMSP	Clinical Genomics Technologies Pty Ltd. (USA)	Sensitivity for recurrence: 75.0% in patients with stage II cancer, 70.6% of the stage III cancers.	NPV= 90.1% (82.1–95.4%) vs. 82.2% (73.7–89.0) for CEA. PPV=61.3% (42.2–78.2%) vs. 60.0% (32.3–83.7) for CEA.					NCT03706235 (C); NCT03706248 (C).	84,85
HCCBloodTest	Liver	Blood	S-ED	*SEPT9* methylation	qMSP	Epigenomics, Inc. (Germany)	76.70% (95% CI: 64.6–85.6)	64.10% (95% CI: 54.5–72.7)		CE-IVD (2018)			NCT03804593 (C).	86
HelioLiver	Liver	Blood	S-ED	Methylation markers (28 target genes, 77 CpG sites) + protein markers	Targeted capture assay	Fulgent Genetics & Helio, Inc. (USA)	85% (95% CI: 78–90) for HCC of any stage; 76% (95% CI: 60–87) for early stage I and II HCC.	91% (95% CI: 85–95)	FDA BDD (2019)		NMPA Acceptance of Registration Application (2021)		NCT05181826 (R); NCT05059665 (C); NCT05053412 (EbI); NCT05199259 (NyR).	49
IvyGene Liver Cancer Test	Liver	Blood	S-ED	ctDNA methylation markers	ddPCR	Laboratory for Advanced Medicine (USA)	80%	86%	FDA BDD (2019)				NCT03694600 (R).	

(Continues)

TABLE 1 (Continued)

epiLiver	Liver	Blood	S-ED	*VASH2*, *CHFR*, *GRID2IP*, *CCNJ*, *F12* methylation	MiSeq NGS	HKG Epitherapeutics (Hong Kong)	84.5%; 75% for early stage.	95%				NCT03483922 (C).	50
Bladder EpiCheck Urine	Bladder and Urothelial	Urine	M-R	15 proprietary methylation markers	Methylation-sensitive restriction enzyme digestion followed by qPCR	Nucleix Ltd. (USA)	68.2% (95% CI: 52.4–81.4). 91.7% (95% CI: 73.0–99.0), excluding Ta-LG patients.	88% (95% CI: 83.9–91.4) NPV = 99.3%; (95% CI: 97.4–99.9), excluding Ta-LG patients.		CE-IVD (2017)			NCT02647112 (C); NCT02700464 (C); NCT04702347 (R).	51–53
UriFind Bladder Cancer Detection Kit	Bladder and Urothelial	Urine	S-ED	*ONECUT2* and *VIM* methylation	qPCR followed by multiplex quantitative PCR	AnchorDx Medical Co., Ltd. (Hong Kong)	91.2% in patients with hematuria. 88.1% in patients with suspected BC.	85.7% in patients with hematuria. 89.7% in patients with suspected BC.	FDA BDD (2021)	CE-IVD (2020)		Guidelines for Diagnosis and Treatment of Bladder Cancer in China (2021)	NCT04948528 (R); (NCT04314245 – Unknown status)	54
EarlyTect Bladder Cancer	Bladder	Urine	S-ED; M-R	*PENK* methylation	LTE-qMSP	Genomictree, Inc. (South Korea)							NCT05220189 (R).	
AssureMDx	Bladder	Urine	S-ED	*TWIST1*, *ONECUT2* and *OTX1* methylation + mutation status of *FGFR3*, *TERT* and *HRAS*	qMSP. SNaPshot™ methylation analysis	MDxHealth (USA)	97%. AUC=0.93 (95% CI: 0.88–0.98)	83%. NPV=99.6–99.9%.					NCT03122964 – Unknown status	55
Bladder CARE	Bladder	Urine	S-ED	*SIM2*, *NKX1-1* and *TRNA-Cys* methylation	Methylation-sensitive restriction enzymes coupled with qPCR	Pangea Laboratory LLC (USA)	93.8%. 90.0% in low grade. 94.1% in high grade.	92.6% PPV = 87.8%; NPV = 96.2%.						56
EsoGuard	Esophagus	Brush cells (Esophageal and gastric)	S-ED	*CCNA1* and *VIM* methylation	NGS	Lucid Diagnostics, Inc. (USA)	88% in esophageal balloon samples. 94.8% in distal esophagus brushings.	91.7% in esophageal balloon samples. 90.5% in distal esophagus brushings.	FDA BDD (2020)	CE-IVD (2021)			NCT04293458 (R); NCT04295811 (R); NCT04880044 (NyR); NCT05210049 (NyR).	57
Oral-M	Esophagus	Oral cell scraping	S-ED	*ZNF582* methylation	qPCR	EpiGene, iStat Biomedical Co., Ltd. (China)				CE-IVD (2016)				58
Epi proLung BL Reflex Assay	Lung	EBUS-TBNA	S-ED	*SHOX2* methylation		Epigenomics, Inc (Germany)				CE-IVD (2010)			NCT01653002 (C).	59,60
Epi proLung	Lung	Blood	S-ED	*SHOX2* and *PTGER4* methylation	qPCR	Epigenomics, Inc (Germany)				CE-IVD (2017)			NCT04321499 (C).	61
PulmoSeek	Lung	Blood	S-ED	100-feature model	Targeted methylation sequencing	AnchorDx Medical Co., Ltd. (Hong Kong)	96.9% (95% CI: 59.4–99.4)	41.7% (95% CI: 35.0–90.0)		CE-IVD (2022)			NCT03651986 (R).	62
QIAsure	Cervix	Conventional exfoliative cytology	S-ED	*FAM19A4* and *hsa-mir124-2* methylation	Multiplex qMSP	Qiagen (Germany)	100% in high-risk HPV+ samples; 67% for CIN3+; 100% for advanced transforming CIN.			CE-IVD (2016)			NCT04111835 (R).	63
Cervi-M	Cervix	Cytology	S-ED	*PAX1* methylation	qPCR	EpiGene, iStat Biomedical Co., Ltd. (China)	> 80% in detection of CIN3 or worse lesions.	> 80% in detection of CIN3 or worse lesions.		CE-IVD (2016)				

TABLE 1 (Continued)

GynTect	Cervix	Liquid-based cytology	S-ED	*ZNF671*, *ASTN1*, *DLX1*, *ITGA4*, *RXFP3*, *SOX17* methylation	qMSP	oncgnostics GmbH (Germany)/ NIM Genetics (New Integrated Medical Genetics) (Spain)	64.8% for CIN3+; 59.7% for CIN2+.	94.6% for CIN3+ PPV=74.5%; NPV=91.7%. 98% for CIN2+ PPV=91.5%; NPV=87.3%).		CE-IVD (2019)			64,65
therascreen PITX2 RGQ	Breast	Tumor	T	*PITX2* methylation	qMSP	Therawis Diagnostics; Qiagen (Germany)				CE-IVD (2018)			66
ConfirmMDx	Prostate	Tumor	S-ED	*GSTP1*, *APC* and *RASSF1* methylation	qMSP	MDxHealth (USA)	62% (95% CI: 51–72).	64% (95% CI: 57–70) NPV = 88% (95% CI: 85-91) - 90% (95% CI: 87-93)			NCCN Prostate Cancer Early Detection (v1.2022). European Association of Urology guidelines (2018)	NCT03082274 – Unknown status.	67,68, 70–72
MGMT Methylation Detection Kit	Glioblastoma	Tumor	T	*MGMT* methylation	qMSP	EntroGen Inc. (USA)				CE-IVD (2018)	NCCN CNS cancers (v1.2022)*		
Human MGMT Gene Methylation Detection Kit	Glioblastoma	Tumor	T	*MGMT* methylation	PAP-ARMS®	Xiamen Spacegen Co., Ltd. (China)				CE-IVD (2016)	NCCN CNS cancers (v1.2022)*		
Therascreen MGMT kit	Glioblastoma	Tumor	T	*MGMT* methylation	Pyrosequencing	Qiagen (Germany)				CE-IVD (2015)	NCCN CNS cancers (v1.2022)*		73
PredictMDx	Glioblastoma	Tumor	T	*MGMT* methylation	qMSP	MDxHealth (USA)					NCCN CNS cancers (v1.2022)*		74
Epicup	Cancer of Unknown Primary	Tumor	CL	Methylation markers	Methylation arrays	Ferrer International (Spain)	97.7% (95% CI: 96.1–99.2)	99.6% (95% CI: 99.5–99.7). PPV=88.6% (95% CI: 85.8–91.3). NPV=99.9% (95% CI: 99.9–100.0).		CE-IVD (2015)			36
OverC Multi-Cancer Detection Blood Test	Multiple	Blood	S-ED	ctDNA methylation markers	NGS (ELSA-seq)	Burning Rock Biotech Ltd. (China)	80.60% (95% CI: 76.0-84.6%) (THUNDER-II trial, ESMO Asia 2020)	98.30% (95%CI: 95.8-99.4%) (THUNDER-II trial, ESMO Asia 2020)		CE-IVD (2022)		NCT04383353 (NyR); NCT04817306 (R); NCT04822792 (R); NCT04820868 (R); NCT04972201 (R).	75
Galleri (GRAIL MCED test)	>50 tumor types	Blood	S-ED	ctDNA methylation markers	Targeted methylation NGS	GRAIL, Inc. (USA)	CCGA trial: 51.50% (95% CI: 49.6–53.3%)	CCGA trial: 99.5% (95% CI: 99.0–99.8). PATHFINDER trial: 99.5%. FPR<1%. PPV=43.1%.	FDA BDD (2019)			NCT02889978 (AnR); NCT03085888 (AnR); NCT04241796 (AnR); NCT05205967 (R).	76–78
PanSeer	Colorectal, Lung, Liver, Stomach, Esophagus	Blood	S-ED	ctDNA methylation markers	NGS	Singlera Genomics Inc. (USA)	83.9–95.7% 3–4 years prior to conventional diagnosis (PCD); 93.6–94.7% 2–3y PCD; 90.5–95.7% 1–2y PCD; 95.2–100% 0–1y PCD.	96.1% (95% CI: 92.5–98.3)				NCT05159544 (R).	79,81
IvyGeneCORE	Breast, Colon, Liver, Lung	Blood	S-ED	ctDNA methylation markers		Laboratory for Advanced Medicine (USA)	84%	90%					

Abbreviations: ACS, American Cancer Society; AnR, active, not recruiting; AUC, area under the receiver operating characteristic curve; BDD, US Food and Drug Administration Breakthrough Device Designation; C, completed; CEA, carcinoembryonic antigen; CE-IVD, in vitro diagnostic (IVD) device that complies with the European In-Vitro Diagnostic Devices Directive 98/79/EC; CI, confidence interval; CIN, cervical intraepithelial neoplasia; CIN2+, cervical intraepithelial neoplasia grade 2 or worse; CIN3+, cervical intraepithelial neoplasia grade 3 or worse; CL, tumor type classifier; CNS, central nervous system; CRC, colorectal cancer; CT, clinical trial; ctDNA, circulating tumor DNA; ddPCR, droplet digital polymerase chain reaction; EbI, enrolling by invitation; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; ESMO Asia 2020, European Society for Medical Oncology Asia Congress 2020; FDA, US Food and Drug Administration; FPR, false-positive rate; HCC, hepatocellular carcinoma; HPV, human papillomavirus; LTE-qMSP, linear target enrichment–quantitative methylation-specific real-time polymerase chain reaction; MCED, multicancer early detection; M-R, monitoring of recurrence; MFDS, Korean Ministry of Food and Drug Safety; MS-HRM, methylation-sensitive high-resolution melting; MSP, methylation-specific polymerase chain reaction; NCCN, National Comprehensive Cancer Network; NGS, next-generation sequencing; NMPA, Chinese National Medical Products Administration; NPV, negative predictive value; NCT ID, ClinicalTrials.gov identifier; NyR, not yet recruiting; PCa, prostate cancer; PCR, polymerase chain reaction; PPV, positive predictive value; qMSP, quantitative real-time methylation-specific polymerase chain reaction; qPCR, quantitative polymerase chain reaction; R, recruiting; sDNA-FIT, stool DNA-fecal immunochemical test; S-ED, screening, early detection; T, treatment response prediction; Ta-LG, low-grade papillary bladder tumors; USPSTF, US Preventive Services Task Force.

[a]In the NCCN CNS cancers guideline, *MGMT* promoter methylation testing is recommended in all high-grade gliomas (grade 3 and 4). There are multiple ways to test for MGMT promoter methylation, including MSP, MS-HRM, pyrosequencing, and ddPCR.

The sections below describe DNA methylation-based assays of clinical utility organized by cancer types, highlighting those that have been FDA-approved and included in renowned guidelines developed by the National Comprehensive Cancer Network (NCCN), the American Cancer Society (ACS), and the US Preventive Services Task Force (USPSTF).

Colorectal cancer

Up to 33 CTs involving DNA-methylation based strategies to detect and/or monitor CRC were identified, 19 of which were related to seven registered DNA-methylation based tests (Figure 3A). A successful example of clinical applications is the development of epigenetic solutions for CRC screening based on the identification of cancer-specific epigenetic alterations in stool DNA. The registered DNA methylation-based assays for CRC screening using stool as analyte include Cologuard (Exact Science Co.), ColoClear (New Horizon Health Technology Company, Ltd.), Earlytect Colon (Genomictree, Inc.), Colosafe (Creative Biosciences [Guangzhou] Company), and Colowell (Shanghai Realbio Technology Company, Ltd.) (Table 1). Cologuard and ColoClear analyze *NDRG4* and *BMP3* methylation plus *KRAS* mutations, whereas the others are based on *SDC2* methylation status. In 2014, Cologuard received full approval from the FDA for adults older than 50 years at average risk of CRC, and the indication was extended to younger individuals (aged ≥45 years) in 2019. By combining an immunochemical assay for human hemoglobin with the molecular genetic and epigenetic analyses, the sensitivity of Cologuard for detecting CRC is significantly superior to the traditional fecal immunochemical test (FIT) (92.3% [95% confidence interval (CI), 83.0%–97.5%] vs. 73.8% [95% CI, 61.5%–84.0%]; $p = .002$), although the specificity among participants with nonadvanced or negative findings is higher in FIT (86.6% [95% CI, 85.9%–87.2%] vs. 94.9% [95% CI, 94.4%–95.3%]; $p < .001$) according to the trial (ClinicalTrials.gov identifier NCT01397747).[46] The use of Cologuard as a CRC screening strategy is recommended by the NCCN Guidelines for CRC Screening (version 2.2022), the ACS CRC screening guideline (2018), and the USPSTF Screening for CRC recommendations (2021). The NCCN and the ACS suggest the same rescreening interval approved by the FDA, every 3 years, whereas the USPSTF recommends testing every 1–3 years.

Another FDA-approved test for CRC screening is the Epi proColon (Epigenomics, Inc.), which is a blood test that analyzes the presence of methylated *SEPT9* in ctDNA.[47,48] By analyzing 1544 samples from prospectively enrolled men and women, aged 50–85 years who were at an average-risk for CRC, the PRESEPT CT (ClinicalTrials.gov identifier NCT00855348) detected a sensitivity for all stages of CRC of 68% (95% CI, 53%–80%), and for stage I–III CRC of 64% (95% CI, 48%–77%), with a specificity of 80% (95% CI, 78%–82%).[48] Moreover, an analysis of paired blood and fecal samples from 290 individuals showed an equivalent sensitivity of Epi proColon (72.2% [95% CI, 62.5%–80.1%]) compared with FIT (68.0% [95% CI, 58.2–76.5] but a lower specificity (80.8% [95% CI, 74.7%–85.8%] vs.

TABLE 2 Investigative clinical trials assessing the performance of previously identified DNA methylation biomarkers

Cancer type	Sample type	Use	Markers	Company/developer	NCT ID	Start year	Country	Study status
Colorectal	Blood	S-ED	ctDNA methylation markers	Fudan University	NCT03737591	2010	China	C
Colorectal	Stool	S-ED	*NPY*, *PENK*, *WIF1* methylation	Assistance Publique, Hopitaux de Paris	NCT01270360	2010	France	C
Colorectal	Stool	S-ED	*VIM*, *BCAT1*, *IKZF1*, *LINE1* methylation	University of Michigan	NCT01511653	2011	USA & Germany	C
Colorectal	Stool	S-ED	Methylation markers (Exact sDNA test)	Case Comprehensive Cancer Center	NCT01647776	2012	USA	C
Colorectal	Stool and blood	S-ED	*BCAT1*, *IKZF1* methylation	Western Sydney Local Health District	NCT02476682	2016	Australia	R
Colorectal	Tumor	P	*LRP1* methylation	CHU de Reims	NCT02786602	2016	France	C
Colorectal	Stool	S-ED	Methylation markers	Sixth Affiliated Hospital, Sun Yat-sen University	NCT03411707	2018	China	R
Colorectal	Blood	M-R	ctDNA methylation markers	Fudan University	NCT03737539	2018	China	R
Colorectal	Stool	DT	*SFRP1* methylation	Xijing Hospital of Digestive Diseases	NCT05204433	2019	China	EbI
Colorectal	Tumor	M-R	Methylation markers (Guangzhou panel, four genes)	Sixth Affiliated Hospital, Sun Yat-sen University	NCT03923335	2019	China	NyR
Colorectal	Blood	M-P	*NPY* methylation	University Hospital, Antwerp	NCT04735900	2020	Belgium	R
Colorectal	Stool	S-ED	Methylation markers	Tri-Service General Hospital	NCT04823793	2021	Taiwan	R
Colorectal	Blood	S-ED	ctDNA methylation markers	Singlera Genomics Inc.	NCT05336539	2022	China	R
Liver	Tumor	P	*VTRNA2-1* methylation	Chang Gung Memorial Hospital	NCT04177316	2018	Taiwan	C
Liver	Blood	M-P, M-R	ctDNA methylation markers	Mayo Clinic	NCT04856046	2021	USA	R
Liver	Blood	DT	Genetron HCC methylation PCR kit (six methylation markers)	Genetron Health	NCT05343832	2021	China	EbI
Cholangiocarcinoma	Brush cells (biliary)	DT	*HOXA1*, *NEUROG1* methylation	Mahidol University	NCT04568512	2016	Thailand	C
Hepatobiliary	Blood	DT	ctDNA methylation markers	Zhujiang Hospital	NCT04835675	2021	China	R
Bladder	Urine	DT, M	*OTX1*, *TWIST1*, *ONECUT2* methylation	Element Diagnostics	NCT02745301	2016	USA	C

(Continues)

TABLE 2 (Continued)

Cancer type	Sample type	Use	Markers	Company/developer	NCT ID	Start year	Country	Study status
Bladder	Urine	DT	*GATA4*, *p16/p14/CDKN2A*, *APC*, *CDH1*, *CD99* methylation	Mansoura University	NCT05362539	2019	Egypt	C
Bladder	Urine	DT	Methylation markers	Creative Biosciences (Guangzhou) Company, Ltd.	NCT05337189	2022	China	R
Bladder and urothelial	Urine	DT	Genetron Uro V1 (*ONECUT2* methyl-ation and mutations in 17 genes)	Genetron Health	NCT04994197	2022	China	R
Gastric	Gastric antrum mucosa	M-R	*MOS* methylation	Seoul National University Hospital	NCT04830618	2012	Korea	C
Gastric	Tumor	M-P	*GFRA1*, *ZNF382* methylation	Beijing Cancer Hospital, Peking University	NCT02159339	2012	China	C
Gastric	Blood	DT	ctDNA methylation markers	Guangzhou Burning Rock Bioengineering Ltd.	NCT05224596	2021	China	R
Gastric	Blood	DT	ctDNA methylation markers and genomic	GeneCast Biotechnology Company, Ltd.	NCT04947995	2021	China	R
Gastric	Blood	DT	ctDNA methylation markers	Singlera Genomics Inc.	NCT05336058	2022	China	R
Gastric	Blood	DM	ctDNA methylation markers	Guangzhou Burning Rock Bioengineering Ltd.	NCT05347524	2022	China	R
Esophagus	Brush cells (esophageal and gastric)	DT	*ZNF682*, *VAV3*, *NDRG4*, *BMP3*, *ZNF568* methylation	Exact Sciences Corporation	NCT02560623	2015	USA	AnR
Esophagus	Brush cells (esophageal and gastric)	DT	*p16*, *NELL1*, *AKAP12*, *TAC1*, *HPP* methylation	National Cancer Institute	NCT04214119	2016	USA	R
Esophagus	Brush cells (esophageal)	M-R	*ZNF345*, *ZNF569*, *TFPI2* methylation plus TFF3 protein expression and *p53* mutation	University of Cambridge, University of Nottingham	NCT04155242	2020	UK	R
Esophagus	Brush cells (esophageal)	DT	EsoCAN assay (three methylation markers)	CapNostics, LLC	NCT05028725	2022	Tanzania & USA	NyR
Nasopharyngeal	Brush cells (nasopharyngeal)	M-R	ctDNA methylation markers	The University of Hong Kong	NCT03379610	2006	China	C
Oral	Oral mucosal biopsy	DT	*p16/CDKN2A* methylation	Peking University	NCT00835341	2005	China	C

TABLE 2 (Continued)

Cancer type	Sample type	Use	Markers	Company/developer	NCT ID	Start year	Country	Study status
Oral	Oral mucosal biopsy	DT	*p16/CDKN2A* methylation	Peking University	NCT01695018	2009	China	C
Oral	Oral mouthwash	DT	*ZNF582*, *PAX1* methylation	National Taiwan University Hospital	NCT01945697	2012	Taiwan	C
Oral	Oral swab	DT	Methylation markers	iStat Biomedical Company, Ltd.	NCT02648789	2015	Taiwan	C
HNSCC	Blood	DT	Methylation signature	Aalborg University Hospital	NCT04567056	2020	Denmark	R
Thyroid	Thyroid needle biopsy	DT	Methylation signature (DDMS-2)	City of Hope Medical Center	NCT05229341	2021	USA	R
Thyroid	Thyroid FNA	DT	Methylation signature (DDMS)	City of Hope Medical Center	NCT03676647	2022	USA	R
Lung	Blood and tumor	P	*p16/CDKN2A*, *DAPK1*, *CDH13*, *APC*, *RASSF1A* methylation	Alliance for Clinical Trials in Oncology	NCT01139944	2010	USA	C
Lung	Blood	DT	ctDNA methylation markers	AnchorDx Medical Company, Ltd.	NCT03181490	2017	China	C
Lung	Blood	DT	ctDNA methylation markers plus mutations	Peking University People's Hospital	NCT03301961	2019	China	R
Lung	Blood	DT	ctDNA methylation markers	EDGC Inc.	NCT04253509	2020	Korea	C
Lung	Blood	DT	ctDNA methylation markers	Palo Alto Veterans Institute for Research	NCT05066776	2021	USA	R
Lung	Sputum	DT	Methylation markers	Creative Biosciences (Guangzhou) Company, Ltd.	NCT05337163	2022	China	R
Lung	Endoscopic cytology supernatant	DT	ctDNA methylation markers (nine genes)	University Hospital, Toulouse	NCT05306912	2022	France	NyR
Cervix	Urine	S-ED	Methylation markers (PreCursor-U+ panel)	Universiteit Antwerpen	NCT04530201	2020	Belgium	R
Cervix	Cervical cytology	S-ED	*EPB41L3*, *JAM3*, *PAX1* methylation	Peking Union Medical College Hospital	NCT04646954	2020	China	R
Cervix	Cervical cytology	S-ED	*PAX1*, *JAM3* methylation	Peking Union Medical College Hospital	NCT05290428	2022	China	R
Breast	RP-FNA	DT	ctDNA methylation markers (seven genes)	University of Texas Southwestern Medical Center	NCT01501656	2012	USA	C

(Continues)

TABLE 2 (Continued)

Cancer type	Sample type	Use	Markers	Company/developer	NCT ID	Start year	Country	Study status
Breast	Blood	T	Multiomic BR(E)2ASTOME algorithm: ctDNA-NGS and gDNA-RRBS	University of Campania "Luigi Vanvitelli"	NCT04996836	2022	Italy	NyR
Endometrium	Blood	DT	*BHLHE22*, *CELF4*, *HAND2*, *ZNF177* methylation	Peking Union Medical College Hospital	NCT04651738	2020	China	R
Endometrium	Cervical cytology	S-ED	*CDO1*, *CELF4* methylation	Beijing Qiyuanjuhe Biotechnology Company, Ltd.	NCT05290415	2022	China	R
Endometrium	Cervical cytology	S-ED	*CDO1*, *CELF4* methylation	Peking Union Medical College Hospital	NCT05290922	2022	China	R
Ovarian	Blood	S-ED	ctDNA methylation markers	RenJi Hospital	NCT03155451	2017	China	C
Ovarian	Blood	DT	*OPCML*, *FODX3*, *CDH13* methylation	Peking Union Medical College Hospital	NCT04651946	2020	China	R
Prostate	Blood and urine	S-ED	*GSTPI*, *CD44*, annexin II, caveolin 1 methylation	National Cancer Institute	NCT00340717	2003	USA	C
Prostate	Blood	DT	Methylation signature (ctMethSig)	University College, London	NCT05020522	2021	UK	NyR
Brain	Blood	M	*p16/CDKN2A*, *p73/TP73*, *MGMT* methylation	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins	NCT00897611	2005	USA	C
Brain	Blood and CSF	CL	Methylation signature	University Health Network, Toronto	NCT04940507	2021	Canada	R
Brain	Tumor	T	*MGMT* methylation	AHS Cancer Control Alberta	NCT04765514	2021	Canada	NyR
Neuroendocrine	Tumor	CL	Methylation signature	University Hospital, Basel	NCT05013957	2021	Switzerland	NyR
Sarcoma	Tumor	T	*MGMT* methylation	National Cancer Institute, Naples	NCT04893356	2021	Italy	AnR
Osteosarcoma	Tumor	T	Methylation markers	Children's Oncology Group	NCT01374672	2011	USA	C
Lymphoma	Blood	DT	ctDNA methylation markers	Guangzhou Burning Rock Dx Company, Ltd.	NCT05275036	2022	China	R
Leukemia	Blood	P	*ITGA4* methylation	Sohag University	NCT05371392	2022	Egypt	NyR

Abbreviations: AHS, Alberta Health Services; AnR, active, not recruiting; C, completed; CHU de Reims, Central Hospital of Reims University; CL, tumor type classifier; CSF, cerebrospinal fluid; ctDNA, circulating tumor DNA; DM, detection of metastasis; DT, diagnosis; EbI, enrolling by invitation; FNA, fine-needle aspiration; gDNA, genomic DNA; HNSCC, head and neck squamous cell carcinoma; M, disease monitoring; M-P, monitoring, predict progression; M-R, monitoring of recurrence, relapse prediction; NCT ID, ClinicalTrials.gov identifier; NGS, next-generation sequencing; NyR, not yet recruiting; P, biomarker of prognosis; PCR, polymerase chain reaction; R, recruiting; RP-FNA, random periareolar fine-needle aspiration; sDNA, stool DNA; S-ED, screening, early detection; RRBS, reduced representation bisulfite sequencing; T, biomarker of treatment, predict response to treatment, or identify actionable targets.

TABLE 3 Exploratory clinical trials focused on identifying DNA methylation biomarkers

Cancer type	Sample type	Use	Markers	Company or developer	NCT ID	Start year	Country	Study status
Colorectal	Stool	DT	Methylation and mutation markers	Exact Sciences Corporation	NCT03789162	2018	USA	R
Colorectal	Stool and blood	DT	Methylation and mutation markers	Exact Sciences Corporation	NCT03821948	2019	USA	C
Liver	Blood	DT	Multiomic, including methylation	Exact Sciences Corporation	NCT03628651	2018	USA & Europe	C
Gastric	Blood and tumor tissue	DT	Methylation markers	Shanghai Zhongshan Hospital	NCT04511559	2020	China	NyR
Gastric	Blood and gastric fluid	T	Methylation and mutation markers	Assistance Publique, Hospitaux de Paris	NCT04253106	2020	France	R
Esophagus	Blood	DT	Methylation markers (eight cancer-related genes)	Johns Hopkins University	NCT00431756	2002	USA	AnR
Oral	Saliva	DT	Multiomic, including methylation	Aalborg University Hospital	NCT04567082	2020	Denmark	R
Lung	Blood	DT	Methylation markers	Exact Sciences Corporation	NCT03633006	2016	USA	C
Lung	Blood	M-PS	Methylation and mutation markers	Peking University People's Hospital	NCT03634826	2018	China	R
Lung	Blood	DT	Methylation markers	Peking University People's Hospital	NCT04558255	2020	China	R
Lung	Blood	DT	Methylation markers	National Taiwan University Hospital	NCT04814407	2021	Taiwan	R
Lung and upper gastrointestinal tract	Rectal mucus	DT	Methylation and mutation markers	Origin Sciences	NCT05102110	2021	UK	NyR
Cervix	Urine and vaginal samples	DT	Multiomic, including methylation	Universiteit Antwerpen	NCT03542513	2017	Belgium	R
Breast	RP-FNA	DT	Methylation markers	National Cancer Institute	NCT00323908	2005	USA	C
Breast	Blood	P	Methylation markers	Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins	NCT00899548	2007	USA	C

(Continues)

TABLE 3 (Continued)

Cancer type	Sample type	Use	Markers	Company or developer	NCT ID	Start year	Country	Study status
Breast	Blood and urine	DT	Multiomic, including methylation	Ospedale Policlinico San Martino	NCT04781062	2021	Italy	R
Endometrium	Tumor tissue	CL	Methylation markers	Gynecologic Oncology Group	NCT01199250	2010	USA	NyR
Ovarian	Blood	DT	Methylation markers	Baylor Research Institute	NCT03622385	2019	USA	AnR
Ovarian	Blood, tumor tissue and uterine lavage	DT	Methylation and mutation markers	Massachusetts General Hospital	NCT04794322	2020	USA	R
Gynecologic[a]	Blood	DT	Multiomic, including methylation	Burning Rock Bioengineering Ltd., Fudan University	NCT04903665	2021	China	R
Prostate	Blood	M-R	Multiomic, including methylation	City of Hope Medical Center	NCT00977457	2007	USA	AnR
Pancreas	Blood	M-PS	Methylation markers	Rigshospitalet, Denmark	NCT04947696	2021	Denmark	R
Adrenocortical	Blood and tumor tissue	DT	Multiomic, including methylation	Children's Oncology Group	NCT01528956	2012	USA	C
Melanoma	Tumor tissue	T	Multiomic, including methylation	ECOG-ACRIN Cancer Research Group	NCT01209299	2012	USA	C
Basal cell carcinoma	Tumor tissue	DT	Methylation markers	Trakya University	NCT04759261	2020	Turkey	R
Sarcoma	Tumor tissue	DT	Multiomic, including methylation	Children's Oncology Group	NCT01466283	2011	USA	C
Sarcoma	Tumor tissue	T	Multiomic, including methylation	Children's Oncology Group	NCT01626170	2012	USA	C
Leukemia	Bone marrow	DT	Methylation markers	Alliance for Clinical Trials in Oncology	NCT00900224	2008	USA	AnR
Leukemia	Bone marrow	DT	Multiomic, including methylation	Children's Oncology Group	NCT01076569	2010	USA	C
Leukemia	Blood	T	Methylation and mutation markers	Eastern Cooperative Oncology Group	NCT01421875	2011	USA	C
Childhood cancer	Tumor tissue	DT	Multiomic, including methylation	Children's Oncology Group	NCT01433224	2011	USA	C
Childhood cancer	Tumor tissue	T	Multiomic, including methylation	Sydney Children's Hospitals Network	NCT03336931	2017	Australia	R

TABLE 3 (Continued)

Cancer type	Sample type	Use	Markers	Company or developer	NCT ID	Start year	Country	Study status
Cancer of unknown primary	Blood and tumor tissue	DT, T	Mutations (future; multiomic)	The Christie NHS Foundation Trust, F. Hoffmann-La Roche Ltd.	NCT04750109	2021	UK	R
Multiple	Blood	DT	Multiomic, including methylation	Exact Sciences Corporation	NCT03662204	2018	USA	AnR
Multiple	Blood	DT	Methylation markers	Nucleix Ltd.	NCT04264767	2019	USA & Israel	AnR
Multiple	Several	DT	Methylation and mutation markers	Centre Hospitalier Universitaire de Besancon	NCT05257707	2022	France	NyR

Abbreviations: AnR, active, not recruiting; C, completed; CL, tumor type classifier; DT, diagnosis; EbI, enrolling by invitation; ECOG-ACRIN, Eastern Cooperative Oncology Group-American College of Radiology Imaging Network; M-PS, monitoring postsurgery; M-R, monitoring of recurrence, relapse prediction; NCT ID, ClinicalTrials.gov identifier; NHS, National Health Service; NyR, not yet recruiting;. P, biomarker of prognosis; R, recruiting; RP-FNA, random periareolar fine-needle aspiration; T, biomarker of treatment, predict response to treatment, or identify actionable targets.

[a]Gynecologic tumors include ovarian, cervical, uterine, vaginal, and vulvar cancers.

97.4% [95% CI, 94.1%–98.9%], respectively). Both methods showed a negative predictive value (NPV) of 99.8%.[69]

In addition to screening and early detection of CRC, epigenetic-based assays can also offer a convenient strategy for monitoring disease recurrence because approximately 30% of patients with stage I–III CRC and up to 65% of patients with stage IV CRC develop recurrent disease after initial treatment.[88] For this purpose, the Colvera assay (Clinical Genomics Technologies Pty Ltd.) has been designed to measure the levels of methylation of *BCAT* and *IKZF1* in plasma. The odds ratio (OR) of recurrence for a positive Colvera test is twice (OR, 14.4; 95% CI, 5.4–38.7; $p < .001$) that for carcinoembryonic antigen (OR, 6.9; 95% CI, 2.3–21.1; $p = .001$), which is the noninvasive biomarker typically used in routine clinical practice for surveillance of disease recurrence. The sensitivity of Colvera for local and distant recurrence are 75% and 66.7%, respectively; compared with 50% and 29.2%, respectively, for carcinoembryonic antigen. In patients who have stage II cancer at diagnosis, the sensitivity of Colvera for recurrence is 75% (70.6% in stage III cancers and 33.3% in stage IV cancers).[84] Colvera also has the potential for identifying residual disease caused by treatment failure. The presence of *BCAT1*-methylated or *IKZF1*-methylated ctDNA after treatment was associated with disease progression (hazard ratio [HR], 9.7; 95% CI, 2.5–37.6) compared with the absence of *BCAT1*/*IKZF1*-methylated ctDNA.[85]

There are also several CTs assessing the performance of previously identified DNA methylation biomarkers for CRC (Table 2). The most recent is a massive community population screening, initiated in 2022, to verify real-world results of a polygene methylation blood test for CRC detection (ClinicalTrials.gov identifier NCT05336539).

Cervical cancer

Simple, noninvasive, highly sensitive tests are needed to increase uptake and adherence rates of population screening programs. Increasing evidence shows that triage of patients using DNA methylation-based assays is a suitable alternative to the well-established invasive methodologies, including for cancer types with well-known risk factors. An example is the screening for cervical cancer. The discovery of the role of the human papillomavirus (HPV) in the initiation and progression of cervical cancer has driven two main actions: first, the screening of HPV-positive women and, second, the development of vaccines against HPV. Although the latter action will continuously decrease the incidence of cervical cancer in those countries with successful vaccination programs, optimization of screening approaches is crucial for accurately identifying women at risk of cervical cancer worldwide. Several studies showing the relevance of epigenetic mechanisms in the neoplastic transformation of precursor premalignant lesions from low-grade (grade 1 cervical intraepithelial neoplasia [CIN1]) to high-grade (CIN3) CIN have supported the use of epigenetic biomarkers to develop in vitro diagnostic medical devices (IVDs). A meta-analysis of 16,336 women in 43 studies showed that DNA methylation assays have higher

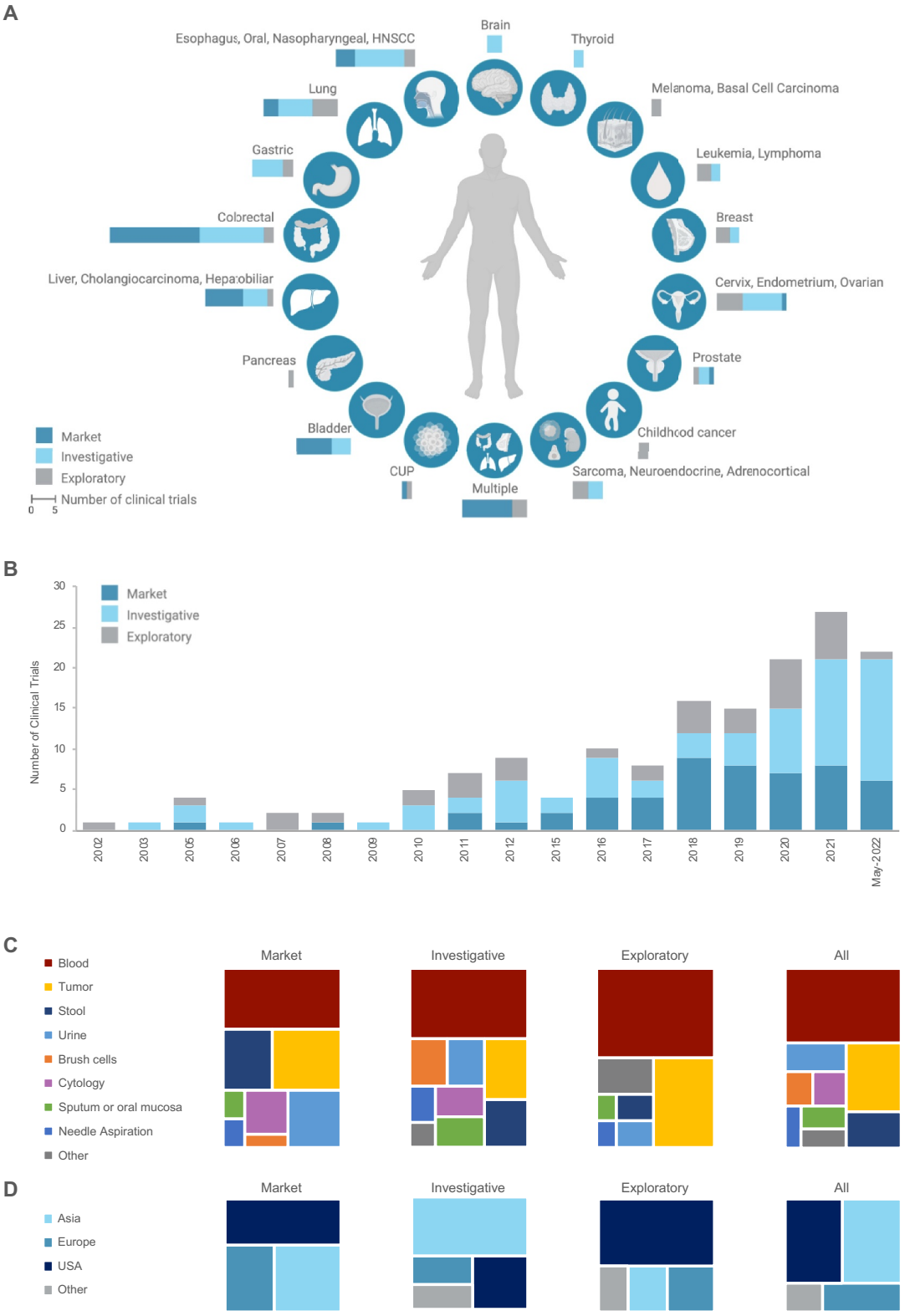

FIGURE 3 Clinical trials (CTs) involving DNA methylation-based strategies. By exploring the ClinicalTrials.gov database as a strategy to identify DNA methylation biomarkers in advanced stages of development for clinical oncology purposes, three categories were established: (1) *market:* CTs designed to determine performance of DNA methylation-based tests that are currently on the market, including CTs that were pivotal in defining biomarker accuracy; (2) *investigative:* CTs designed to assess the performance of previously identified DNA methylation biomarkers; and (3) *exploratory:* CTs aimed at identifying DNA methylation biomarkers. Distributions of CTs according to (A) tumor type, (B) start year, (C) sample type, and (D) geographic region are depicted (A was created with BioRender.com). CUP indicates cancer of unknown primary; HNSCC, head and neck squamous cell carcinoma.

specificity than HPV16/HPV18 genotyping or cytology of atypical squamous cells of undetermined significance or greater (≥ASCUS) as a triage test.[89] Among women in whom high-risk HPV (hrHPV) genotypes have been detected in cervical specimens (hrHPV-positive), the relative sensitivity of DNA methylation assays for the detection of ≥CIN2 was 1.22 (95% CI, 1.05–1.42) compared with HPV16/HPV18 genotyping, and it was 0.81 (95% CI, 0.63–1.04) compared with cytology of ≥ASCUS; whereas the relative specificity was 1.03 (95% CI, 0.94–1.13) and 1.25 (95% CI, 0.99–1.59), respectively. Importantly, DNA methylation assays provide an advantage over HPV16/HPV18 genotyping because they are not restricted to the detection of ≥CIN2 associated only with HPV16/HPV18. Moreover, testing can be performed using the same clinician-collected or self-collected sample used for HPV screening. There are three European Compliance (CE)-certified DNA methylation-based tests: QIAsure (QIAGEN), Cervi-M (iStat Biomedical Company, Ltd.), and GynTect (oncgnostics GmbH; Table 1). QIAsure analyzes the methylation status of *FAM19A4* and hsa-mir124-2, and can detect >98% of cervical cancers, irrespective of histology type, International Federation of Gynecology and Obstetrics stage (FIGO), sample type, and HPV genotype.[63,90] Therefore, even challenging cases beyond those that are hrHPV-positive, such as rare histotypes (including clear cell carcinomas, neuroendocrine carcinomas, and hrHPV-negative cervical carcinomas), can be screened with this assay. The use of objective molecular biomarker tests with a high positive predictive value (PPV) and a high NPV for ≥CIN2 or ≥CIN3, such as *FAM19A4/miR124-2* methylation, could reduce the number of colposcopy referrals without loss of clinical sensitivity to detect cervical cancer and advanced CIN.[63,90] The second assay, Cervi-M, uses *PAX1* methylation as an auxiliary biomarker for cervical cancer screening and is able to detect >80% of ≥CIN3 lesions. *PAX1* methylation has been associated with the transition of CIN1 to CIN2/CIN3 and from CIN2/CIN3 to cervical cancer.[91] The third, the GynTect test analyzes *DLX1*, *ITGA4*, *RXFP3*, *SOX17*, and *ZNF671*, whose hypermethylation has been correlated with the presence of cervical precancerous lesions and cervical cancer.[65] The use of these assays, alone or in combination with cytology, could prevent unnecessary colposcopy referrals and better guide surveillance strategies.

There are also ongoing CTs aimed at confirming the potential of previously identified DNA methylation biomarkers for cervical cancer (Table 2). One of them is the massive METHY3 study (ClinicalTrials.gov identifier NCT04646954) to validate the preliminary results of the METHY1 trial (ClinicalTrials.gov identifier NCT03961191). The METHY1 study (n = 306 patients) not only demonstrated that the diagnostic accuracy of *EPB41L3* and *JAM3* methylation is comparable with that of hrHPV-based strategies but also found that positive methylation is able to differentiate ≥CIN2 from inflammation/CIN1 in cases with negative hrHPV results.[92] The METHY3 trial plans to screen 12,000 cases to confirm the robustness of the combined analysis of *EPB41L3* and *JAM3* methylation as an hrHPV-independent predictor of the risk of cervical cancer.

Hepatocellular carcinoma

Preexisting cirrhosis is found in the vast majority of individuals diagnosed with hepatocellular carcinoma (HCC), thus screening and surveillance for HCC is considered cost effective in patients with cirrhosis of any cause, including hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol, and nonalcoholic fatty liver disease, but also in patients with chronic hepatitis B even in the absence of cirrhosis.[93] The 5-year survival rate is >70% in patients who have early stage HCC, and the median survival is 12–18 months for those with symptomatic, advanced-stage disease, supporting the importance of HCC surveillance in high-risk individuals. Nevertheless, surveillance by imaging with or without using alpha-fetoprotein as a biomarker remains suboptimal for early stage HCC detection.[94,95] DNA methylation-based IVDs are gaining ground in HCC screening, and two of them have already received FDA Breakthrough Device Designation (BDD): the HelioLiver test (Fulgent Genetics & Helio, Inc.) and the IvyGene Liver Cancer Test (Laboratory for Advanced Medicine; Table 1). Importantly, the HelioLiver test reaches a sensitivity of 76% (95% CI, 60%–87%) for early (stage I and II) HCC and a specificity of 91% (95% CI, 85–95) by combining methylation (77 CpG sites) and protein markers.[49] A similar sensitivity for detecting early stage HCC has been reported for the epiLiver test (HKG Epitherapeutics) using methylation of CpGs residing in the *VASH2*, *CHFR*, *GRID2IP*, *CCNJ*, and *F12* genes. epiLiver classifies patients with HCC at 95% specificity and 84.5% sensitivity and detects 75% of patients with early stage A disease.[50] There is also a CE-IVD, the HCCBloodTest (Epigenomics, Inc.), which uses *SEPT9* methylation as a biomarker to detect HCC. The HCCBloodTest has a sensitivity of 76.70% (95% CI, 64.6%–85.6%) and a specificity of 64.10% (95% CI, 54.5%–72.7%[86]; Table 1). All of these IVDs for HCC detection have been designed to capture methylation events in plasma-derived ctDNA, facilitating their use as a screening strategy.

Bladder cancer

Urine is another body fluid that can provide molecular information with valuable clinical utility. It is particularly useful for detecting bladder cancer. Because hematuria can be an early sign of bladder cancer, but only 3%–28% of patients with hematuria are diagnosed with bladder cancer, accurate screening of patients with hematuria is critical. Several epigenetic-based assays in urine samples have been developed as less invasive and inexpensive alternatives to cystoscopy to assess the risk of bladder cancer for patients with hematuria. Of these, UriFind (AnchorDx Medical Company, Ltd.) obtained FDA BDD in July 2021. UriFind, based on the dual-marker detection of *ONECUT2* and *VIM* methylation, had 91.2% sensitivity and 85.7% specificity in patients with hematuria and had 88.1% sensitivity and 89.7% specificity in patients with suspected bladder cancer (Table 1). Importantly, this assay has shown better sensitivities than cytology and fluorescence in situ hybridization (FISH) for detecting noninvasive low-grade papillary bladder (Ta) tumors (UriFind, 83.3%

sensitivity in patients with hematuria and 83.3% in patients with suspected bladder cancer vs. 22.2%–41.2% for cytology and 44.4%–52.9% for FISH) and nonmuscle-invasive bladder cancer (NMIBC; UriFind, 80.0%–89.7% vs. 51.5%–52.0% for cytology and 59.4%–72.0% for FISH) with comparable specificities.[54] Another inherent issue in NMIBC is the high recurrence rates of up to 50%–70% after 5 years, which means that patients require lifelong postoperative surveillance. The use of urinary markers rather than invasive cystoscopy simplifies surveillance schedules. Several lines of evidence support the clinical utility and influence on decision making of the CE-certified Bladder EpiCheck test (Nucleix Ltd.) in the surveillance of NMIBC.[51–53] This assay, consisting of 15 proprietary DNA methylation biomarkers, has a sensitivity of 68.2% (95% CI, 52.4%–81.4%) and of 91.7% (95% CI, 73.0%–99.0%) if low-grade Ta tumors are excluded, and has a specificity of 88% (95% CI, 83.9%–91.4%[51]) (Table 1).

Esophageal cancer

Analysis of DNA methylation markers can also be useful for detecting precancerous lesions, such as Barrett esophagus (BE), a premalignant condition of the distal esophagus that increases the risk of esophageal cancer. Detection of BE currently requires esophagogastroduodenoscopy, an invasive and expensive procedure that is not routinely used. The DNA methylation-based EsoGuard assay (Lucid Diagnostics, Inc.) overcomes these limitations by analyzing BE-specific hypermethylation events in *CCNA1* and *VIM* in esophageal brush cells collected using a swallowable balloon device.[57] The high sensitivity (88%) and specificity (91.7%) of this simple, minimally invasive strategy using DNA from nonendoscopic balloon sampling of the distal esophagus make EsoGuard a suitable alternative for BE screening.[57] Moreover, sensitivity in BE with high-grade dysplasia was 100% in 23 distal esophagus brushings, and it was 50% in four esophageal balloon samples.[57] This CE-IVD received the FDA BDD in 2020 (Table 1).

Prostate cancer

The high sensitivity of epigenetic biomarkers also makes them an ideal strategy for guiding the detection of occult PCa. False-negative rates of prostate biopsy procedures reach 10%–30%, mainly because of sampling error.[96] Although multiparametric magnetic resonance imaging-guided biopsies have reduced the problem of false-negative biopsies, accurate methods to better identify the patients most likely to benefit from repeat biopsy after an initial negative biopsy are needed. ConfirmMDx (MDxHealth) is a tissue-based test that analyzes the methylation status of *GSTP1*, *APC*, and *RASSF1*, genes frequently methylated in PCa. Therefore, methylation status of these genes in PCa-negative biopsies is used to guide physician decision making about repeating a prostate biopsy. High NPVs for ConfirmMDx have been reported by two independent studies: 90% (95% CI, 87%–93%) in the Methylation Analysis to Locate Occult Cancer (MATLOC) trial[67] and 88% (95% CI, 85%–91%) in the Detection of Cancer Using Methylated Events in Negative Tissue (DOCUMENT) study.[70] Moreover, both studies identified the epigenetic assay as an independent predictor of patient outcome (MATLOC: OR, 3.17; 95% CI, 1.81–5.53; DOCUMENT: OR, 2.69; 95% CI, 1.60–4.51[67,70]) (Table 1). Although ConfirmMDx has not been approved by the FDA, it is included in the NCCN Guidelines for Prostate Cancer Early Detection (version 1.2022) among the tests that improve specificity in the postbiopsy setting that should be considered in patients thought to be at higher risk despite a negative prostate biopsy.

Glioblastoma

Based on robust studies demonstrating that *MGMT* methylation is an independent predictor of a favorable response of gliomas to alkylating agents, such as carmustine (BCNU) or temozolomide,[97–99] several epigenetic assays have been commercialized to predict response to alkylating chemotherapy. The MGMT Methylation Detection Kit (EntroGen Inc.), the Human MGMT Gene Methylation Detection Kit (Xiamen Spacegen Company, Ltd.), and the Therascreen MGMT kit (QIAGEN) are among the CE-certified assays (Table 1). The NCCN Guidelines for CNS Cancers (version 1.2022) recommends *MGMT* promoter methylation testing in all grade III and IV gliomas. The type of tests included in the NCCN recommendation are quantitative methylation-specific PCR, methylation-specific high-resolution melting, pyrosequencing, and ddPCR.

Breast cancer

PITX2 methylation predicts outcome to adjuvant anthracycline-based chemotherapy in patients with high-risk (lymph node-positive, estrogen receptor [ER]-positive, HER2-negative) breast cancer.[100,101] The Therascreen PITX2 RGQ PCR assay (QIAGEN) is a CE-marked test with high reliability and robustness for determining *PITX2* promoter methylation status and for predicting the outcomes after anthracycline-based chemotherapy in patients with high-risk breast cancer (HR, 2.48; $p < .001$).[66]

Cancer of unknown primary

The intrinsic tissue specificity of epigenomic profiles has also been exploited to develop DNA methylation-based cancer type classifiers. Clinical applications include the identification of tumor origin in CUP, a heterogeneous group of metastatic tumors for which a standardized diagnostic work-up fails to identify the site of primary origin at the time of diagnosis.[102,103] This limitation seriously hinders clinical management and treatment decision making. EPICUP (Ferrer International) is a classifier of cancer type based on microarray DNA methylation signatures that can predict the primary cancer by

analyzing the CUP biopsy to guide more precise therapies associated with better outcomes[36,104] (Table 1). This cancer type classifier showed 99.6% specificity (95% CI, 99.5%–99.7%), 97.7% sensitivity (95% CI, 96.1%–99.2%), 88.6% PPV (95% CI, 85.8%–91.3%), and 99.9% NPV (95% CI, 99.9%–100.0%) in a validation set of 7691 tumors. EPICUP predicted a primary cancer of origin in 188 of 216 patients (87%) with CUP, and those who received a tumor type-specific therapy showed improved overall survival (OS) compared with patients who received empiric therapy (HR, 3.24; 95% CI, 1.42–7.38; $p = .0051$).[36]

Multicancer early detection tests

Another application of DNA methylation-based cancer type classifiers is the development of multicancer early detection (MCED) tests using ctDNA methylation markers, such as the OverC Multi-Cancer Detection Blood Test (Burning Rock Biotech Ltd.), the Galleri test (GRAIL Inc.),[76–78] PanSeer (Singlera Genomics Inc.), and the IvyGeneCORE test (Laboratory for Advanced Medicine; Table 1).

The Galleri test obtained the FDA BDD in 2019. According to the Circulating Cell-free Genome Atlas study (ClinicalTrials.gov identifier NCT02889978), this test has a specificity for cancer signal detection of 99.5% (95% CI, 99.0%–99.8%) and an overall sensitivity of 51.5% (95% CI, 49.6%–53.3%; stage I, 16.8% [95% CI, 14.5%–19.5%]; stage II, 40.4% [95% CI, 36.8%–44.1%]; stage III, 77.0% [95% CI, 73.4%–80.3%]; stage IV, 90.1% [95% CI, 87.5%–92.2%]).[77] To assess implementation of the Galleri test into clinical practice, the PATHFINDER study[78] (ClinicalTrials.gov identifier NCT04241796) tested 6662 individuals aged 50 years or older. According to recent results, the Galleri test detected a cancer signal in 92 participants, and the cancer diagnosis was confirmed in 35 of 92 patients (38%). Importantly, 25 of 35 patients (71%) were diagnosed with cancer types that have no routine cancer screening available. The refined version of Galleri test (MCED-Scr), which reduced the detection of premalignant hematologic conditions, had a specificity of 99.5%, a false-positive rate <1%, and a PPV of 43.1%. The cancer signal origin prediction to identify cancer type had an accuracy of 97%.

PanSeer is another MCED test and currently is available for research use only. This assay detects five common types of cancer (colorectal, lung, liver, stomach, and esophageal cancers) up to 4 years earlier than the current standard of care.[81] In total, 1379 randomly selected samples from the Taizhou Longitudinal Study were used to train and test the PanSeer assay. According to preliminary results from 605 asymptomatic individuals, 191 of whom were later diagnosed with any of the five cancer types within 4 years of blood draw, PanSeer sensitivity 3–4 years before conventional diagnosis (PCD) was 83.9%–95.7%; (2–3 years PCD, 93.6–94.7%; 1–2 years PCD, 90.5%–95.7%; 0–1 years PCD, 95.2%–100%). Specificities of 94.7% (95% CI, 90.7%–97.3%) and 96.1% (95% CI, 92.5%–98.3%) have been reported in training and test sets, respectively.[81]

DNA methylation-based cancer classifiers for CNS tumors and sarcomas

The epigenetic characterization of human tumors has revealed characteristic methylation patterns that can also be used to develop molecular classifiers, thereby providing an additional tool for more precise diagnosis that can improve clinical management. The impact of DNA methylation-based tumor classification is clear from its inclusion in the World Health Organization (WHO) classification of adult[105] and pediatric[106] CNS tumors. DNA methylation profiling has refined and reshaped the landscape of CNS tumor classification. Nearly all CNS tumor types are aligned to a distinctive methylation signature,[31] and the current edition of the WHO CNS tumor classification now includes information about diagnostic methylation profiling as *essential and desirable diagnostic criteria* that can provide more critical guidance for diagnosis of particular CNS tumor types/subtypes.[105] DNA methylation-based CNS tumor classification is being implemented in several institutions worldwide as a diagnostic tool that complements conventional histopathologic approaches.[107,108] The added value of this strategy in the diagnosis of challenging pediatric CNS tumors[109] further corroborates the relevant contribution of epigenomics to the clinical oncology.

The DNA methylation-based CNS classification system developed by the German Cancer Consortium (DKFZ)[31] is available online for research purposes (https://www.molecularneuropathology.org). On this free web platform, unprocessed .IDAT files of Human Methylation 450K or EPIC BeadChip arrays can be uploaded and automatically compared with a reference cohort >2800 neuropathologic tumors of almost all known entities (80 tumor classes or subclasses are currently included) to obtain the brain tumor methylation classifier result that could aid in clinical decision making. In a parallel analysis with standard histopathologic approaches performed in >1000 CNS tumors, the DKFZ DNA-methylation based classification was in accordance with the pathologic diagnosis in 76% of cases ($n = 838$). However, it changed the diagnosis in 12% of prospective cases ($n = 129$), of which several were IDH wild-type astrocytomas and anaplastic astrocytomas that were reclassified as IDH wild-type glioblastomas. This demonstrates the substantial impact of DNA methylation-based classification on diagnostic precision compared with standard methods, and it could serve as a blueprint for other tumor types.[31]

Although current strategies are based on the analysis of CNS tumor biopsies, the high risk of invasive procedures to access brain tumors has encouraged the development of novel strategies, such as blood-based liquid biopsies. However, efficient passage of tumor biomarkers into the peripheral circulation is hindered by the blood–brain barrier. A recent strategy to overcome this limitation, which has yielded promising preclinical results,[110] is being tested in the recently initiated BRAINFUL trial (ClinicalTrials.gov identifier NCT04940507; Table 2).To enhance the release of tumor DNA into the circulation to improve the detection of DNA methylation signatures, magnetic resonance-guided focused ultrasound is used as a strategy to transiently disrupt the blood–brain barrier. Moreover, the positive

correlation observed between DNA methylation profiles obtained from cerebrospinal fluid samples and tumor tissues from pediatric patients with medulloblastoma opens new avenues for exploring the use of cerebrospinal fluid as a source of ctDNA for DNA methylation profiling not only to detect tumor occurrence and define subtype but also to monitor treatment response and tumor recurrence.[111]

DNA methylation is also playing a major role in improving the classification of sarcomas. Although distinctive molecular alterations (mostly translocations that generate gene fusions) guide the diagnosis of many sarcoma types, approximately one half of sarcoma entities lack unequivocal genomic hallmarks.[32] A DNA methylation-based classification tool for soft tissue and bone sarcomas representing a broad range of subtypes and age groups has recently been developed.[32] Methylation-based diagnostic assignment of undifferentiated tumors with small blue round cell histology also illustrates the power of this strategy for precisely classifying challenging tumors.[33] Validation of other DNA methylation-based cancer classifiers in large cohorts of patients is expected over the next few years, and this will broaden the range of tools available for more precise cancer diagnosis and thereby improve clinical management.

Other investigative and exploratory clinical trials involving DNA methylation biomarkers

In addition to the 53 CTs related to registered DNA methylation-based tests (Table 1), 67 trials assessing the clinical performance of previously identified DNA methylation biomarkers have been launched (Table 2), of which 28 (42%) have been initiated since 2021 (Figure 3B), illustrating the accelerated advance of the clinical epigenetics field. An area of major development is the use of epigenetic approaches for population screening, mainly associated with the possibility of obtaining tumor-derived DNA from body fluids (Figure 2). The use of liquid biopsies as a surrogate of tumor tissue is being broadly extended in the epigenetic field (Figure 3C). Most of the ongoing trials are testing ctDNA methylation markers in plasma for early cancer detection or for monitoring recurrence or progression. An example is a massive community population screening to verify real-world results of a polygene ctDNA methylation detection test for CRC that has recently begun (ClinicalTrials.gov identifier NCT05336539).

Even so, exfoliative cytology samples are still the samples of choice to screen for cervical and esophageal cancers, urine is the sample of choice to screen for bladder cancer, and stool is the sample of choice to screen for CRC (Figure 3C). For instance, the trials EC-METHY2 (ClinicalTrials.gov identifier NCT05290415) and EC-METHY3 (ClinicalTrials.gov identifier NCT05290922) propose to recruit a total of 17,000 cases to evaluate the accuracy of *CDO1* and *CELF4* methylation in cervical cytology samples for endometrial cancer screening based on results from the EC-METHY trial (ClinicalTrials.gov identifier NCT04651738). These trials seek to provide a profound basis for the approval of this DNA methylation-based assay for endometrial screening in China. Another example is a clinical study to assess the performance of a multigene methylation detection kit for lung cancer detection in sputum that has just started (ClinicalTrials.gov identifier NCT05337163).

Regarding exploratory studies, Table 3 summarizes the identified CTs designed to discover novel DNA methylation biomarkers for clinical oncology. Most of them not only investigate DNA methylation profiles but also perform multiomic analyses to explore different layers of molecular information. For instance, the PROMEO trial (ClinicalTrials.gov identifier NCT04567082) is comparing proteomic and methylation profiles in saliva from patients who have oropharyngeal cancer with those from a control population, with the aim of identifying robust biomarkers for noninvasive diagnosis. There is also a study focused on developing an early detection test for gynecologic malignancies (including ovarian, cervical, uterine, vaginal, and vulvar cancers) by liquid biopsy in peripheral blood using biomarkers of cfDNA methylation, ctDNA mutation, and proteins (the PERCEIVE-I trial; ClinicalTrials.gov identifier NCT04903665). Recently reported preliminary results showed that the methylation model was superior to the protein model in identifying gynecologic malignancies (sensitivity, 72.4% [95% CI, 64.0%–79.8%] vs. 56.8% [95% CI, 47.9%–65.4%]), especially in the early stages (stage I: sensitivity, 46.0% [95% CI, 31.8%–60.7%] vs. 26.5% [95% CI, 15.0%–41.1%]; stage II: sensitivity, 79.2% [95% CI, 57.9%–92.9%] vs. 39.1% [95% CI, 19.7%–61.5%]); with comparable specificity (99.0% [95% CI, 96.3%–99.9%] vs. 99.4% [95% CI, 98.0%–99.9%]).[112] A similar multiomic approach combining the analysis of multiple circulating biomarkers from blood (ctDNA, proteins, exosomes) and urine (ctDNA) with radiomics is being used to develop a Horizontal Data Integration classifier for the diagnosis of early stage breast cancer (ClinicalTrials.gov identifier NCT04781062).[113] These studies could provide novel epigenetic-based health care solutions in the near future. The distribution of DNA methylation-associated CTs in the United States, Europe, Asia, and other regions for the three categories (market, investigative, and exploratory) is shown in Figure 3D.

Histone modifications as biomarkers in cancer

Solid preclinical studies have revealed an aberrant landscape of histone modifications in cancer (reviewed by Zhau et al.[12]). A global loss of monoacetylation and trimethylation of histone H4, particularly lysine 16 (K16) acetylation (H4K16ac) and K20 trimethylation (H4K20me3), is a common hallmark of human cancer cells. These changes are associated with the hypomethylation of DNA repetitive sequences.[114] Moreover, alterations in the methylation patterns of H3K9 and H3K27 have been associated with aberrant gene silencing in many cancers,[115] and global histone modification patterns have been suggested as predictors of prognosis in various cancer types[116–119] as well as predictors of the risk of PCa recurrence.[120] Phosphorylation of histone H3 at serine 10 (H3S10ph), a mark involved in proliferation and transcriptional activation, has been recognized as an important player in cancer initiation and dissemination. An increase in this mark has been associated with a poor prognosis in several tumor types.[121] The clinical use

of histone modification changes as biomarkers in cancer is still under development, but a promising example that could be useful in the clinical setting is the use of a histone mark as an ancillary diagnostic tool for distinguishing melanoma from an unusual proliferative nodule in children.[122] Although the majority of proliferative nodules that develop during childhood from congenital melanocytic nevi are benign, and melanoma development is a rare event, the distinction of melanoma from a proliferative nodule is a clinical and histopathologic challenge. The detection of H3K27me3 expression in a benign proliferative nodule, in contrast to significant loss or complete lack of expression in nodular melanomas of childhood associated with congenital melanocytic nevi, as evidenced by immunohistochemistry staining, could provide an additional tool to improve diagnosis.[122]

Efforts are also being made to develop minimally invasive approaches to assess histone modifications. A recently described method to capture circulating nucleosomes in plasma and quantify their associated histone modifications[123] provides a way of performing liquid biopsies to characterize cancer-specific histone marks. Moreover, recurrent oncogenic somatic mutations of histone genes, also known as *oncohistones*, occur across cancer types, including glioma, sarcoma, and lymphoma.[124] These mutations alter the epigenome by provoking the functional inhibition of the cognate histone writer, leading to disruption of the epigenetic and transcriptomic state. Although oncohistones are not epigenetic biomarkers per se, the case of H3K27M mutation merits description, considering its implications for diagnosis and the impact on epigenetic PTMs. High-throughput sequencing techniques revealed a unique clinical and biologic subtype of pediatric diffuse intrinsic pontine gliomas characterized by a mutation of K27 in the H3.3 histone variant (H3K27M).[125,126] Considering the crucial role of PTMs of H3K27 in gene expression, this mutation invokes the disruption of methylation and acetylation patterns that could potentially alter the expression of oncogenes and tumor suppressor genes. Importantly, H3K27M mutation in H3.3 is universally associated with short survival in diffuse intrinsic pontine glioma, in contrast to improved survival in patients harboring wild-type H3.3 (mean OS, 0.73 $\pm$ 0.48 years for patients with H3K27M-mutated tumors vs. 4.59 $\pm$ 5.55 years for patients with wild-type tumors; $p = .0008$). Moreover, in a multivariate analysis that included age, histologic diagnosis, and H3.3 mutation status, H3.3 mutation status was the only significant predictor of OS (HR, 4.3; 95% CI, 1.3–14.5; $p = .019$).[127] The significant prognostic and therapeutic implications have led to the inclusion of a novel entity called *diffuse midline glioma, H3K27M-mutant* in the 2016 revision of the WHO CNS tumor classification as a grade IV distinct entity, carrying a fatal prognosis.[128]

The histone field of greatest interest from a clinical perspective is the histone modifiers, which are the enzymes responsible for introducing the histone PTMs. An emblematic example is EZH2, which is the enzymatic subunit of polycomb repressive complex 2 (PRC2), a complex that methylates H3K27 to promote transcriptional silencing. Gain-of-function and loss-of-function mutations in *EZH2* have been described in several cancer types. Moreover, overexpression of EZH2 is a marker of advanced and metastatic disease in many solid tumors, including PCa and breast cancer.[129] Strong evidence demonstrating the role of EZH2 as a cancer driver has prompted the development of EZH2-specific inhibitors (EZH2i),[130] as described below. Some CTs assessing the efficacy of EZH2i analyze levels of H3K27me3 in blood before and after treatment, with the aim of evaluating the effect of escalated doses and of exploring the use of H3K27me3 as a biomarker of disease response (ClinicalTrials.gov identifiers NCT03603951, NCT03854474, NCT02601950, and NCT04390737).

EPIGENETIC DRUGS

The fundamental role of epigenetic mechanisms in shaping genome function, coupled with the epigenetic dysregulation that occurs in cancer, have made the epigenetic machinery an attractive target for drug development, particularly given the plasticity of the epigenetic modifications. Therefore, the development of *epidrugs* (drugs that target the enzymes involved in epigenetic regulation of genome function) as a strategy for tackling cancer is an active field of research. Current epigenetic drugs target enzymes that introduce (writers), recognize (readers), and remove (erasers) epigenetic marks to DNA or core histones (Figure 4A). The rationale behind the use of epigenetic drugs lies in the possibility of restoring a balanced transcriptional landscape by modifying the chromatin states. An overview of key molecular mechanisms responsible for antitumor effects of epigenetic drugs is provided in Figure 5, featuring the pleiotropic effects that hinder cancer progression mainly through cell cycle arrest, cell differentiation, and apoptosis, as well as the immunomodulatory properties of the epidrugs to restore the antitumor immune response. Considering the scope of this review, the sections below describe epigenetic drugs approved by the FDA and several epidrugs that are being assessed in CTs. Approved indications for epidrugs and inclusions in the NCCN Guidelines, as well as information about drug efficacy from pivotal CTs, are summarized in Table 4.[131–161]

DNA methyltransferase inhibitors

The first-generation DNMT inhibitor (DNMTi) azacitidine (5-azacytidine; Vidaza; Celgene Corporation) and decitabine (5-aza-2′-deoxycytidine; Dacogen; Otsuka America Pharmaceutical, Inc.) are pyrimidine analogues that are incorporated into DNA during replication, where they create irreversible covalent DNA-DNMT adducts leading to dual epigenetic and cytotoxic effects. Covalent trapping and proteasome-mediated degradation of DNMTs result in the passive loss of cytosine methylation in the daughter cells after replication, whereas adducts trigger the activation of DNA damage response and can ultimately lead to apoptosis, particularly at high concentrations. DNA hypomethylation and direct cytotoxicity on

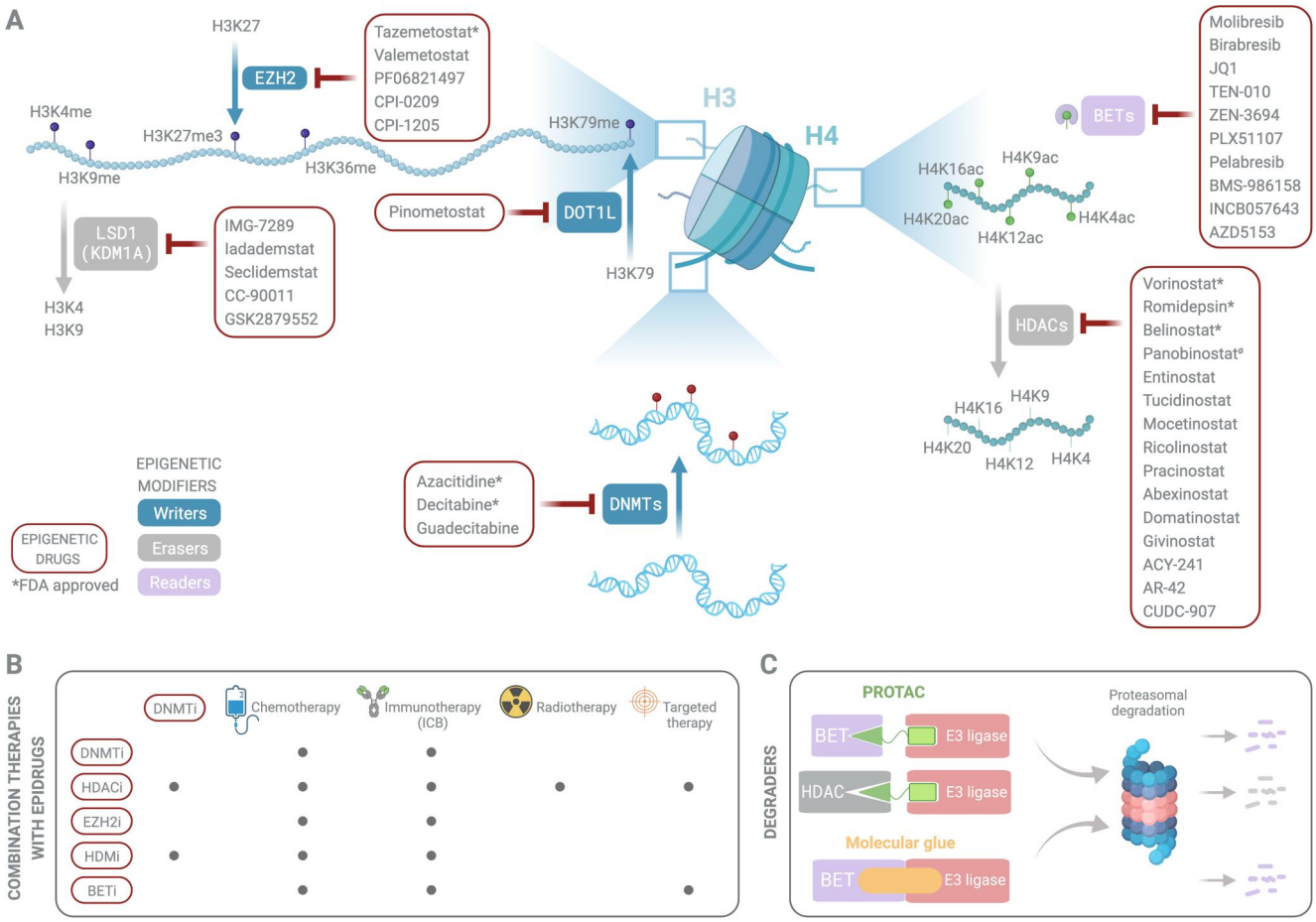

FIGURE 4 Epigenetic drugs for cancer treatment. The reversibility of epigenetic modifications makes epigenetic machinery an attractive target for drug development. (A) Inhibitors for targeting epigenetic writers, such as DNA methyltransferases (DNMTs) and histone methyltransferases EZH2 and DOT1L, epigenetic erasers, such as histone demethylase (HDM) LSD1/KDM1A and histone deacetylases (HDACs), and epigenetic readers, such as bromodomain and extraterminal domain (BET) proteins, have been developed. Epigenetic drugs (epidrugs) already approved by the US Food and Drug Administration (FDA; *) and those entered into clinical trials are listed. The revoked FDA approval of panobinostat is denoted with the symbol ø. The mechanisms of action of epidrugs through inhibition of epigenetic enzymes are depicted as red horizontal lines. A simplified representation is shown because, for instance, BET proteins also recognize acetylated lysines in the H3 tail. (B) Combination treatments of epidrugs with chemotherapy, immunotherapy (immune checkpoint blockage [ICB]), radiotherapy, and targeted therapy that have entered into clinical trials are indicated. (C) Proteolysis-targeting chimeras (PROTACs) and molecular glues to degrade HDACs and BET proteins have been designed by hijacking the intracellular ubiquitin proteasome system. ac indicates acetylation; BETi, BET protein inhibitor; DOT1L, DOT1-like histone lysine methyltransferase; DNMTi, DMNT inhibitor; EZH2, enhancer of zeste homolog 2; EZH2i, EZH2 inhibitor; H3, histone 3; H4, histone 4; HDACi, HDAC inhibitor; HDMi, HDM inhibitor; K, lysine; LSD1, lysine-specific histone demethylase (also known as KDM1A); me, methylation; me3, trimethylation. Figure created with BioRender.com.

abnormal, rapidly dividing tumor cells could be responsible for their antineoplastic effects[162] (Figure 5). In addition to DNA, azacitidine incorporates mainly into RNA, induces ribosomal disassembly, and prevents translation, impairing normal cellular processes. This increases the side effects because azacitidine can affect not only rapidly dividing cancer cells but also normal cells in the course of their proper cell cycle. Moreover, both azacitidine and decitabine are chemically unstable drugs that undergo rapid and spontaneous hydrolysis in aqueous solution and are deaminated by cytidine deaminase, which hinders their clinical applications.[162] However, accumulated preclinical evidence demonstrating that DNMTis are able to restore altered DNA methylation and gene expression profiles has encouraged the development of CTs.

Azacitidine and decitabine for the treatment of hematologic malignancies: Myelodysplastic syndrome, acute myeloid leukemia, and juvenile myelomonocytic leukemia

With an overall response rate of 15.7% in the azacitidine treatment group ($n = 99$) versus a lack of response in the observation group ($n = 92$; $p < .0001$), azacitidine was the first agent approved by the FDA for the treatment of myelodysplastic syndrome (MDS) in 2004.[131] This was followed by the approval of decitabine in 2006, supported by an ORR of 17% in patients who had MDS and received decitabine ($n = 89$) compared with supportive care (ORR, 0%; $n = 81$; $p < .001$).[138] In both cases, approval was for administration as an

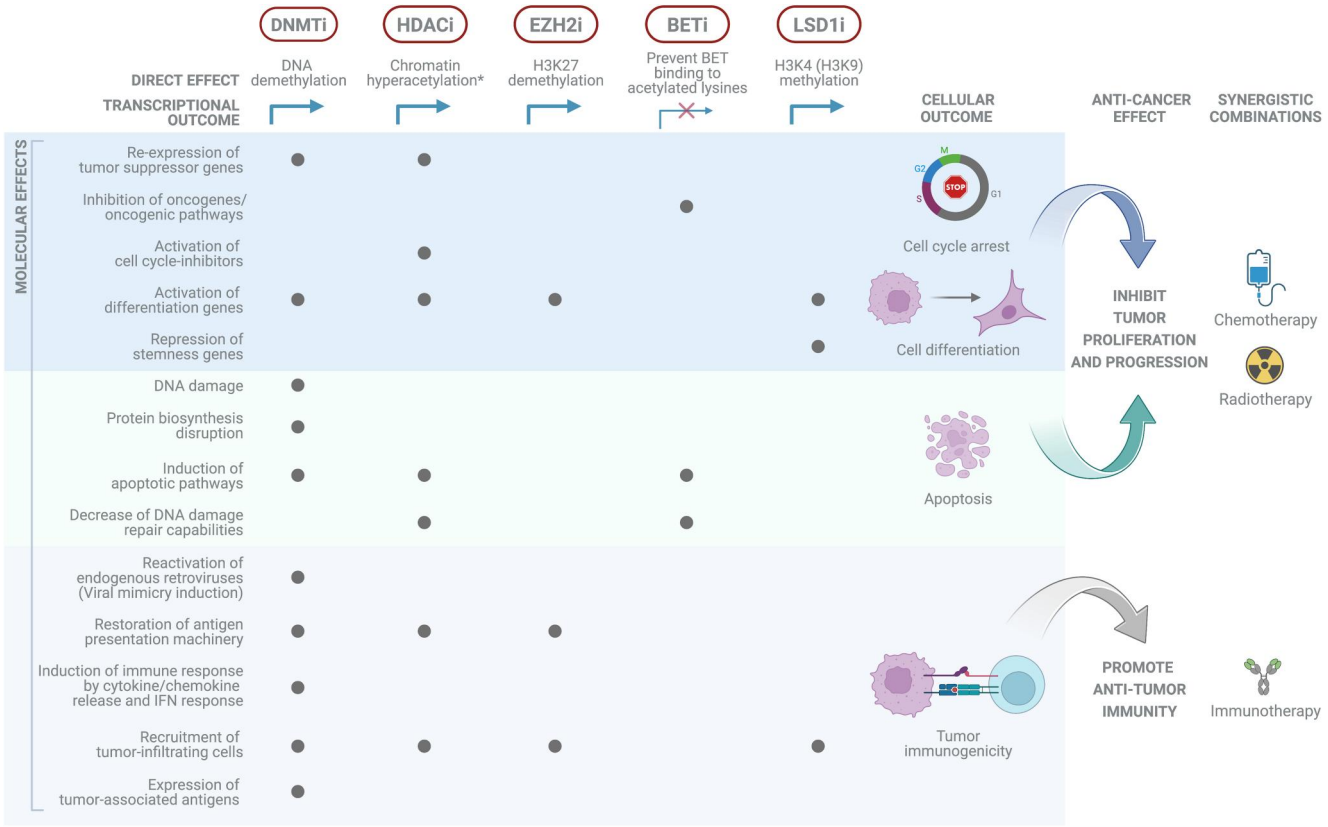

FIGURE 5 Molecular mechanisms responsible for antitumor effects of epigenetic drugs. Epigenetic drugs could restore a balanced transcriptional landscape by modifying the chromatin states. The effects of inhibitors of DNMTs (DNMTi), HDACs (HDACi), EZH2 (EZH2i), BET proteins (BETi), and LSD1 (LSD1i) are summarized here, featuring the pleiotropic mechanisms that hinder cancer progression mainly through cell cycle arrest, cell differentiation, and apoptosis, as well as the immunomodulatory properties of the epidrugs to restore the antitumor immune response. The mechanisms behind the anticancer activity of epidrugs are also the rationale behind drug combinations with other therapies, such as chemotherapy, radiotherapy, and immunotherapy. *HDACs also have multiple nonhistone substrates, so the impact of HDAC inhibition is extended to other proteins, including several transcription factors. BET indicates bromodomain and extraterminal domain; DNMT, DNA methyltransferase; EZH2, enhancer of zeste homolog 2; H3, histone 3; HDAC, histone deacetylase; IFN, interferon; K, lysine; LSD1, lysine-specific histone demethylase 1. In the Transcriptional Outcome, the thick blue arrow indicates transcriptional activation, whereas the blue arrow crossed by a red X depicts transcriptional silencing. Figure created with BioRender.com.

injectable suspension. In 2020, the FDA approved the oral combination of decitabine and cedazuridine (Inqovi; Otsuka Pharmaceutical Co.) for patients with MDS, based on complete remission (CR) rates of 18% (95% CI, 10%–28%) in the ASTX727-01-B trial (ClinicalTrials.gov identifier NCT02103478; $n = 80$) and 21% (95% CI, 15%–29%) in the ASTX727-02 (ClinicalTrials.gov identifier NCT03306264; $n = 133$) trial.[139,140] All of these indications are included in the NCCN Guidelines for MDS (version 1.2023) (Table 4).

The combination of the BCL-2 antagonist venetoclax with azacitidine or decitabine in elderly patients with previously untreated AML received accelerated FDA approval in 2018, supported by the CRs observed in 25 patients treated with azacitidine (CR rate, 37%; 95% CI, 26%–50%) and in seven patients treated with decitabine (CR rate, 54%; 95% CI, 25%–81%) in the M14-358 trial (ClinicalTrials.gov identifier NCT02203773).[132,133] The regular FDA approval for this indication was granted in 2020, supported by improvements in OS (14.7 months [95% CI, 11.9–18.7 months] vs. 9.6 months [95% CI, 7.4–12.7 months]) and in the CR rate (37% [95% CI, 31%–43%] vs. 18% [95% CI, 12%–25%]) in the venetoclax plus azacitidine group ($n = 286$) compared with placebo plus azacitidine ($n = 145$), as reported by the VIALE-A study (ClinicalTrials.gov identifier NCT02993523).[134] These indications are included in the NCCN Guidelines for AML (version 2.2022) (Table 4).

In 2020, the FDA approved azacitidine tablets (ONUREG, CC-486; Celgene Corporation) for continued treatment of patients with AML in CR, based on results from the QUAZAR trial (ClinicalTrials.gov identifier NCT01757535), which reported a median OS of 24.7 months (95% CI, 18.7–30.5 months) in the ONUREG arm ($n = 238$) versus 14.8 months (95% CI, 11.7–17.6 months) in the placebo arm ($n = 234$; HR, 0.69; 95% CI, 0.55–0.86; $p < .001$).[137] The NCCN Guidelines for AML (version 2.2022) also include this indication (Table 4). Combination treatments with the oral azacitidine ONUREG are being assayed in CTs. A phase 2/3 trial comparing standard drug therapy (R-miniCHOP: rituximab combined with low-dose cyclophosphamide, doxorubicin, vincristine, and prednisone) alone or in combination with ONUREG is ongoing in patients with newly diagnosed diffuse large B cell lymphoma (DLBCL) older than 75 years (ClinicalTrials.gov identifier NCT04799275). Moreover, the

TABLE 4 Epigenetic drugs in clinical practice

Drug	MOA	Company (commercial name)	Route	FDA-approved indications (year of approval)	Guidelines[a]	Other approvals or designations	Drug efficacy in clinical trials	Selected ongoing clinical trials for drug combinations
Azacitidine	DNMTs inhibition	Pharmion Corporation, Celgene Corporation (Vidaza)	Intravenous	MDS (2004): All MDS subtypes	NCCN Guidelines for MDS (v1.2023)		ORR = 15.7% in the azacitidine group (n = 99) vs. 0.0% in the observation group (n = 92); (p < .0001; Kaminskas 2005[131])	
				AML (2018, 2020): Venetoclax in combination with azacitidine; patients with newly diagnosed AML (aged ≥75 years) or who have comorbidities precluding intensive induction chemotherapy	NCCN Guidelines for AML (v2.2022)		M14-358 trial (NCT02203773): CR in 25 patients (37.0%; 95% CI, 26.0%–50.0%; DiNardo 2018,[132] 2019[133]) VIALE-A trial (NCT02993523): mOS = 14.7 months (95% CI, 11.9–18.7 months) and CRR = 36.7% (95% CI, 31.0%–43.0%) in the venetoclax plus azacitidine group (n = 286) vs. mOS = 9.6 months (95% CI, 7.4–12.7 months) and CRR = 17.9% (95% CI, 12.0%–25.0%) in the placebo plus azacitidine group (n = 145). (HR, 0.66 [95% CI, 0.52–0.85]; p < .001 for mOS; DiNardo 2020[134])	
				IDH1-mutated AML (2022): Ivosidenib in combination with injectable azacitidine: patients with newly diagnosed AML (aged ≥75 years) who have a susceptible *IDH1* mutation or who have comorbidities precluding intensive induction chemotherapy	NCCN Guidelines for AML (v2.2022)		AG120-C-009 trial (NCT03173248): mOS = 24.0 months (95% CI, 11.3–34.1 months) and CRR = 47.0% (95% CI, 35.0%–59.0%) in the ivosidenib plus azacitidine arm (n = 72) vs. mOS = 7.9 months (95% CI, 4.1–11.3 months) and CRR = 15.0% (95% CI, 8.0%–25.0%) in the placebo plus azacitidine arm (HR, 0.44 [95% CI, 0.27–0.73]; p = .0010 for mOS; Montesinos 2022[135])	

TABLE 4 (Continued)

Drug	MOA	Company (commercial name)	Route	FDA-approved indications (year of approval)	Guidelines[a]	Other approvals or designations	Drug efficacy in clinical trials	Selected ongoing clinical trials for drug combinations
				JMML (2022): Pediatric patients with newly diagnosed JMML			AZA-JMML-001 trial (NCT02447666): Clinical response in 9 patients (50.0%; 95% CI, 26.0%–74.0%, including cCR in 3 patients and cPR in 6 patients (Niemeyer 2021[136])	
Oral azacitidine	DNMT inhibition	Celgene Corporation (Onureg)	Oral	AML (2020): Continued treatment of patients with AML who achieve first CR or CRi after intensive induction chemotherapy and are not able to complete intensive curative therapy	NCCN Guidelines for AML (v2.2022)		QUAZAR trial (NCT01757535): mOS = 24.7 months (95% CI, 18.7–30.5 months) in the Onureg arm (n = 238) and 14.8 months (95% CI, 11.7–17.6 months) in the placebo arm (n = 234); (HR, 0.69 [95% CI, 0.55–0.86]; p < .001; Wei 2020[137])	
Decitabine	DNMT inhibition	MGI PHARMA, Inc. (Dacogen)	Intravenous	MDS (2006): MDS patients including previously treated and untreated, de novo, and secondary MDS, and intermediate-1, intermediate-2, and high-risk IPSS groups.	NCCN Guidelines for MDS (v1.2023)		ORR = 17.0% in decitabine group (n = 89) vs. 0.0% in supportive care group (n = 81); (p < .001; Kantarjian 2006[138])	
				AML (2018, 2020): Venetoclax in combination with decitabine: patients with newly diagnosed AML (aged ≥75 years) or who have comorbidities precluding intensive induction chemotherapy	NCCN Guidelines for AML (v2.2022)		M14-358 trial (NCT02203773): CR in 7 patients (54.0%; 95% CI, 25.0%–81.0%); CR + CRi rate of 67% for venetoclax in combination with azacitidine or decitabine (DiNardo 2018,[132] 2019[133])	
		Otsuka Pharmaceutical Company (Inqovi)	Oral	MDS (2020): Oral combination of decitabine and	NCCN Guidelines for MDS (v1.2023)		ASTX727-01-B trial (NCT02103478; n = 80): CRR = 18.0% (95% CI,	

(Continues)

TABLE 4 (Continued)

Drug	MOA	Company (commercial name)	Route	FDA-approved indications (year of approval)	Guidelines[a]	Other approvals or designations	Drug efficacy in clinical trials	Selected ongoing clinical trials for drug combinations
				cedazuridine (Inqovi); adult patients with MDS, including previously treated and untreated, de novo, and secondary MDS, and intermediate-1, intermediate-2, and high-risk IPSS groups			10.0%–28.0%); ASTX727-02 trial (NCT03306264; n = 133): CRR = 21.0% (95% CI, 15.0%–29.0%); (Garcia-Manero 2020,[139] Kim 2022[140])	
Vorinostat	HDAC inhibition	Merck & Company, Inc. (Zolinza)	Oral	CTCL (2006): Patients with CTCL who had progressive, persistent, or recurrent disease or after two systemic therapies	NCCN Guidelines for Primary Cutaneous Lymphomas (v2.2022)		Study I (n = 74), ORR = 30.0% (95% CI, 19.7%–41.5%); study II (n = 33), ORR = 31.0% (95% CI, 9.1%–61.4%); (Mann 2007[141])	Dual combination of vorinostat with pembrolizumab in advanced prostate, renal, or urothelial tumors (phase 1; NCT02619253); DLBCL, FL, or Hodgkin lymphoma (phase 1; NCT03150329); NSCLC (phase 1/2; NCT02638090); and squamous cell carcinoma (phase 2; NCT04357873)
Romidepsin	HDAC inhibition	Gloucester Pharmaceuticals, Inc. (Istodax)	Intravenous	CTCL (2009): Patients with CTCL who have received at least one prior systemic therapy	NCCN Guidelines for Primary Cutaneous Lymphomas (v2.2022)		ORR = 34.0% (24 of 71 patients; 95% CI, 23.0%–46.0%; Piekarz 2009[142]); and ORR = 34.0% (33 of 96 patients; 95% CI, 25.0%–45.0%; Whittaker 2010[143])	
		Celgene Corporation (Istodax)		PTCL (2011), but FDA approval was withdrawn as of May 9, 2022	NCCN Guidelines for T-Cell Lymphomas (v2.2022)		ORR = 25.0% (33 of 130 patients; Coiffier 2012[144]); FDA withdrawal of approval based on lack of improved PFS, response rates, and overall survival in the NCT01796002 trial (Bachy 2022[145])	

TABLE 4 (Continued)

Drug	MOA	Company (commercial name)	Route	FDA-approved indications (year of approval)	Guidelines[a]	Other approvals or designations	Drug efficacy in clinical trials	Selected ongoing clinical trials for drug combinations
Belinostat	HDAC inhibition	Spectrum Pharmaceuticals, Inc. (Beleodaq)	Intravenous	PTCL (2014): R/R PTCL	NCCN Guidelines for T-Cell Lymphomas (v2.2022)		ORR = 25.8% (95% CI, 18.3%–34.6%), CRR = 10.8% (95% CI, 5.9%–17.8%), PRR = 15.0% (95% CI, 9.1%–22.7%), $n = 120$ patients (Lee 2015[146])	
Panobinostat	HDAC inhibition	Novartis Pharmaceuticals Corporation (Farydak)	Oral	MM (2015), but FDA approval was withdrawn as of March 24, 2022			PANORAMA1 trial (NCT01023308): Panobinostat plus bortezomib and dexamethasone group ($n = 387$) vs. placebo plus bortezomib and dexamethasone group ($n = 381$); PFS = 11.99 months (95% CI, 10.33–12.94 months) vs. 8.08 months (95% CI, 7.56–9.23 months; HR, 0.63 [95% CI, 0.52–0.76]; $p < .0001$; San-Miguel 2014[147]); in November 2021 Secura Bio, Inc., requested withdrawal of approval for Farydak because it was not feasible for them to complete the required postmarketing clinical trials	
Tucidinostat	HDAC inhibition	Chipscreen Biosciences Ltd. (Chidamide)				China FDA approval (2014): R/R PTCL	ChiCTR-TNC-10000811 trial: ORR = 28.0% (22 of 79 patients); PFS = 2.1 months (range, from 1 day to 44.9 months); OS = 21.4 months (range, 0.3–50.1 months); (Shi 2015[148]); results led to CFDA approval of tucidinostat in R/R PTCL	
						China FDA approval (2019): Tucidinostat plus exemestane: postmenopausal	ACE trial (NCT02482753): mPFS = 7.4 months (95% CI, 5.5–9.2 months) in the tucidinostat plus	

(Continues)

TABLE 4 (Continued)

Drug	MOA	Company (commercial name)	Route	FDA-approved indications (year of approval)	Guidelines[a]	Other approvals or designations	Drug efficacy in clinical trials	Selected ongoing clinical trials for drug combinations
						patients with advanced, hormone receptor-positive breast cancer	exemestane group and 3.8 months (95% CI, 3.7–5.5 months) in the placebo group (HR, 0.75; 95% CI, 0.58–0.98; $p = .033$; Jiang 2019[149]); results led to CFDA approval of tucidinostat plus exemestane for postmenopausal patients with advanced, hormone receptor-positive breast cancer	
Entinostat	HDAC inhibition	Syndax Pharmaceuticals				FDA BTD (2013): Entinostat in combination with exemestane: ER-positive advanced breast cancer	ENCORE 301 trial (NCT00676663): mOS = 28.1 months in exemestane plus entinostat (EE) group ($n = 64$) vs. 19.8 months in exemestane plus placebo (EP) group ($n = 66$); (HR, 0.59; 95% CI, 0.36–0.97; $p = .036$; Yardley 2013[150]) However, randomized phase 3 trial E2112 (NCT02115282): mPFS = 3.3 months (EE) vs. 3.1 months (EP); (HR, 0.87; 95% CI, 0.67–1.13; $p = .30$); mOS = 23.4 months (EE) vs. 21.7 months (EP) (HR, 0.99; 95% CI, 0.82–1.21; $p = .94$); ORR = 5.8% (EE) and 5.6% (EP); (Connolly 2021[151])	Entinostat with immunotherapeutic agents in NSCLC, melanoma, and CRC (phase 1b/2; ENCORE-601; NCT02437136), metastatic renal cell carcinoma (phase 2; NCT03552380), melanoma (phase 2; NCT03765229), bladder cancer (phase 2; NCT03978624), cholangiocarcinoma and pancreatic cancer (phase 2; NCT03250273), lymphoma (phase 2; NCT03179930), MDS (phase 1; NCT02936752), and several advanced solid tumors (phase 1; NCT02909452)

TABLE 4 (Continued)

Drug	MOA	Company (commercial name)	Route	FDA-approved indications (year of approval)	Guidelines[a]	Other approvals or designations	Drug efficacy in clinical trials	Selected ongoing clinical trials for drug combinations
Tazemetostat	EZH2 inhibition	Epizyme, Inc. (Tazverik)	Oral	FL (2020): R/R FL in adult patients with *EZH2*-mutated tumors who have received at least two prior systemic therapies and for R/R FL in adult patients who have no satisfactory alternative treatment options	NCCN Guidelines for B-Cell Lymphomas (v5.2022)		NCT01897571: ORR = 69.0% (95% CI, 53.0%–82.0%; 31 of 45 patients) in the *EZH2*-mutated cohort vs. 35.0% (95% CI, 23.0%–49.0%; 19 of 54 patients) in the *EZH2* wild-type cohort (Morschhauser 2020[152])	Tazemetostat with immune checkpoint blockage: pembrolizumab for HNSCC (phase 1/2; NCT04624113) and urothelial tumors (phase 1/2; NCT03854474); and durvalumab for multiple solid tumors (phase 2; NCT04705818)
				Epithelioid sarcoma (2020): Patients aged ≥16 years with metastatic or locally advanced epithelioid sarcoma not eligible for complete resection	NCCN Guidelines for Soft Tissue Sarcoma (v2.2022)		NCT02601950: ORR = 15.0% (95% CI, 7.0%–26.0%; 9 of 62 patients; Gounder 2020[153])	Tazemetostat plus doxorubicin in the front-line setting in epithelioid sarcoma (phase 3; NCT04204941)
Valemetostat	EZH2 inhibition	Daiichi Sankyo (Ezharmia)	Oral			Japanese MHLW approval (2022): R/R ATL	NCT04102150: ORR = 48.0% (90% CI, 30.5%–65.9%; 12 of 25 patients), CRR = 20.0% (5 of 25 patients), PRR = 28.0% (7 of 25 patients); (Izutsu 2022[154])	Valemetostat with ipilimumab in patients with metastatic prostate, urothelial, or renal cell cancer (phase 1; NCT04388852)
Iadademstat	LSD1 inhibition	Oryzon Genomics SA				FDA ODD for AML (2021) and SCLC (2022)	EudraCT No.: 2013-002447-29; phase 1 trial in R/R AML: one CR (Salamero 2020[155])	ALICE Trial (EudraCT 2018-000482-36): Iadademstat with azacitidine for the treatment of AML, ORR = 73% (5 CR, 6 CRi, and 5 PR); (Salamero 2021[156]); CLEPSIDRA (EudraCT no 2018-000469-35; phase 2): Iadademstat in combination with platinum-etoposide for SCLC treatment

(Continues)

TABLE 4 (Continued)

Drug	MOA	Company (commercial name)	Route	FDA-approved indications (year of approval)	Guidelines[a]	Other approvals or designations	Drug efficacy in clinical trials	Selected ongoing clinical trials for drug combinations
Seclidemstat	LSD1 inhibition	Salarius Pharmaceuticals, Inc.				FDA FTD for R/R Ewing sarcoma (2019)		Seclidemstat as monotherapy or in combination with topotecan and cyclophosphamide in patients who have R/R Ewing sarcoma or Ewing-related sarcomas with FET-family translocations (phase 1/2; NCT03600649)
Enasidenib	*IDH2*-mutant inhibition	Celgene Corporation (Idhifa)	Oral	AML (2017): Adult patients with R/R AML who have a susceptible *IDH2* mutation	NCCN Guidelines for AML (v2.2022)		AG221-C-001 trial (NCT01915498): ORR = 40.3% (95% CI, 33.0%–48.0%; 71 of 176 patients), including CR in 34 patients (19.3%; 95% CI, 13.8%–25.9%); (Stein 2017[157])	Enasidenib in combination with azacitidine (phase 2; NCT03683433), venetoclax (NCT04092179; phase 1/2), or ruxolitinib (phase 2; NCT04281498) in *IDH2*-mutated patients with R/R AML or MPN
Ivosidenib	*IDH1*-mutant inhibition	Agios Pharmaceuticals/ Servier Pharmaceuticals LLC (Tibsovo)	Oral	*IDH1*-mutated AML (2018): Adult patients with R/R AML who have a susceptible *IDH1* mutation	NCCN Guidelines for AML (v2.2022)		AG120-C-001 trial (NCT02074839): ORR = 41.6% (95% CI, 32.9%–50.8%; 52 of 125 patients), with CR or CRi in 38 patients (ORR = 30.4%; 95% CI, 22.5%–39.3%); (DiNardo 2018[158])	Ivosidenib in combination with nivolumab (Ivo-Nivo) for *IDH1*-mutant advanced solid tumors (phase 2; NCT04056910); ivosidenib with mFOLFIRINOX in resectable pancreatic adenocarcinoma (phase 1; NCT05209074)

TABLE 4 (Continued)

Drug	MOA	Company (commercial name)	Route	FDA-approved indications (year of approval)	Guidelines[a]	Other approvals or designations	Drug efficacy in clinical trials	Selected ongoing clinical trials for drug combinations
				IDH1-mutated AML (2019): Adult patients with newly diagnosed AML who are aged ≥75 years or who have comorbidities precluding intensive induction chemotherapy	NCCN Guidelines for AML (v2.2022)		AG120-C-001 trial (NCT02074839): CR + CRh rate = 42.4% (14 of 33 patients; 95% CI, 25.5%–60.8%); CRR = 30.3% (10 of 33 patients; 95% CI, 15.6%–48.7%); (Roboz 2020[159])	
				IDH1-mutated cholangiocarcinoma (2021): Adult patients with previously treated, locally advanced or metastatic cholangiocarcinoma who have an *IDH1* mutation	NCCN Guidelines for Hepatobiliary Cancers (v2.2022)		ClarIDHy trial (AG120-C-005, NCT02989857): mPFS = 2.7 months (95% CI, 1.6–4.2 months) with ivosidenib vs 1.4 months (95% CI, 1.4–1.6 months) with placebo (HR, 0.37; 95% CI, 0.25–0.54; one-sided $p < .0001$); mOS = 10.3 months (95% CI, 7.8–12.4 months) with ivosidenib vs 7.5 months (95% CI, 4.8–11.1 months) with placebo (HR, 0.79; 95% CI, 0.56–1.12; one-sided $p = .09$); (Zhu 2017[160])	
				IDH1-mutated AML (2022): Ivosidenib in combination with injectable azacitidine or as monotherapy: patients with newly diagnosed AML aged ≥75 years who have a susceptible *IDH1* mutation or who have comorbidities	NCCN Guidelines for AML (v2.2022)		AG120-C-009 trial (NCT03173248): mOS = 24.0 months (95% CI, 11.3–34.1 months) and CRR = 47.0% (95% CI, 35.0%–59.0%) in the ivosidenib plus azacitidine arm ($n = 72$) versus 7.9 months (95% CI, 4.1–11.3 months) and CRR = 15.0% (95% CI, 8.0%–25.0%) in the placebo plus	

(Continues)

TABLE 4 (Continued)

Drug	MOA	Company (commercial name)	Route	FDA-approved indications (year of approval)	Guidelines[a]	Other approvals or designations	Drug efficacy in clinical trials	Selected ongoing clinical trials for drug combinations
				precluding intensive induction chemotherapy			azacitidine arm (HR, 0.44; 95% CI, 0.27–0.73; $p = .0010$ for mOS); (Montesinos 2022[135])	
					NCCN Guidelines for Bone Cancer (v1.2023): *IDH1*-mutant conventional or dedifferentiated chondrosarcoma		AG120-C-002 trial (NCT02073994): mPFS = 5.6 months (95% CI, 1.9–7.4 months); PFS at 6 months, 39.5%; SD in 11 of 21 patients (52%); (Tap 2020[161])	

Abbreviations: AML, acute myeloid leukemia; ATL, adult T-cell leukemia/lymphoma; BTD, Breakthrough Therapy US Food and Drug Administration Designation; cCR, clinical complete remission; CFDA, China Food and Drug Administration; CI, confidence interval; CMML, chronic myelomonocytic leukemia; cPR, clinical partial remission; CR, complete remission; CRC, colorectal cancer; CRh, complete remission with partial hematologic improvement; CRi, complete remission with incomplete count recovery; CRR, complete remission rate; CTCL, cutaneous T-cell lymphoma; DLBCL, diffuse large B-cell lymphoma; DNMT, DNA methyltransferase; ER, estrogen receptor; *EZH2*, enhancer of zeste 2; FDA, US Food and Drug Administration; FL, follicular lymphoma; mFOLFIRINOX, modified chemotherapy with combined folinic acid, 5-flurouracil, irinotecan, and oxaliplatin; FTD, Fast Track US Food and Drug Administration Designation; HDAC, histone deacetylase; HR, hazard ratio; HNSCC, head and neck squamous cell carcinoma; *IDH1*, isocitrate dehydrogenase 1; *IDH2*, isocitrate dehydrogenase 2; IPSS, International Prognostic Scoring System; JMML, juvenile myelomonocytic leukemia; LSD1, lysine-specific histone demethylase 1; MDS, myelodysplastic syndrome; MHLW, Ministry of Health, Labor, and Welfare in Japan; MM, multiple myeloma; MOA, mechanism of action: mOS, median overall survival; mPFS, median progression-free survival; MPN, myeloproliferative neoplasms; NCCN, National Comprehensive Cancer Network; NCT, National Clinical Trial identifier (ClinicalTrials.gov ID); NSCLC, nonsmall cell lung cancer; ODD, Orphan Drug US Food and Drug Administration Designation; ORR, overall response rate; OS, overall survival; PFS, progression-free survival; PRR, partial remission rate; PTCL, peripheral T-cell lymphoma; R/R, relapsed or refractory; SCLC, small cell lung cancer; SD, stable disease.

[a]Detailed indications are available in NCCN guidelines.

OMNIVERSE phase 1 study is evaluating the safety, tolerability, and preliminary efficacy of ONUREG in combination with venetoclax in newly diagnosed, relapsed and/or refractory (R/R) AML (ClinicalTrials.gov identifier NCT04887857).

In 2022, azacitidine (Vidaza) was granted FDA approval for pediatric patients with newly diagnosed juvenile myelomonocytic leukemia based on results from the AZA-JMML-001 trial (ClinicalTrials.gov identifier NCT02447666), in which a clinical response was detected in nine patients (50%; 95% CI, 26%–74%), including clinical CR or partial remission (PR) in three and six patients, respectively[136] (Table 4).

Additional DNMT inhibitors and promising combinations

Despite the significant benefits of azacitidine and decitabine in the clinical management of several hematologic malignancies, unfavorable pharmacokinetic and pharmacodynamics, lack of target selectivity, and off-target effects of these inhibitors, as well as the lack of benefits in solid tumors, has forced the development of second-generation DNMTi agents with superior pharmacokinetic and pharmacodynamic properties. One of these agents is guadecitabine, a prodrug of decitabine with chemical improvements to increase resistance to degradation by cytidine deaminase, prolonging half-life and exposure of cancer cells to the active metabolite. Although the phase 3 studies ASTRAL-2 (ClinicalTrials.gov identifier NCT02920008) and ASTRAL-3 (ClinicalTrials.gov identifier NCT02907359) in patients with previously treated AML and MDS or chronic myelomonocytic leukemia have failed to demonstrate the superiority of guadecitabine compared with alternative treatment,[163] the potential of DNMTi agents in combination therapies is becoming clear.

Much effort is currently being made to explore the power of epidrugs as modulators of sensitivity to other antineoplastic therapies (Figure 4B), and this is opening up new opportunities for using them in solid tumors. In this regard, epigenetic drugs have the potential to reverse many processes in which tumors engage to evade immune-mediated destruction. The use of combinatory therapies with epidrugs might help overcome some of the current limitations of immunotherapy.[164] The synergistic action of the epigenetic drugs could be related to the activation of genes that are silenced in cancer cells, including tumor surface antigens, endogenous retroviruses, and proteins from the major histocompatibility complex, whose re-expression could increase tumor visibility to the host immune system (Figure 5). The potent immunomodulatory activity of DNMTi agents on host immune cells as well as cancer cells[165–167] has promoted the development of CTs to assess the ability of DNMTi agents to improve the clinical benefits of immune checkpoint inhibitors. Several ongoing CTs are testing the combination of guadecitabine with anti-PD1 (nivolumab, pembrolizumab) or anti-PD-L1 (durvalumab, atezolizumab) in solid tumors, including lung cancer, melanoma, renal cell carcinoma, and urothelial tumors (ClinicalTrials.gov identifiers NCT03308396, NCT04250246, NCT03220477, NCT03257761, NCT02998567, NCT03179943, and NCT03206047).

Moreover, in contrast to the classical pyrimidine analogs, such as azacitidine, decitabine, or guadecitabine, which covalently bind and irreversibly inhibit the activity of all three canonical DNMTs (maintenance DNMT1 and de novo DNMT3A and DNMT3B); a first-in-class, nonnucleoside, reversible, and selective DNMT1 inhibitor has recently been described.[168] GSK3685032 competes with the active-site loop of DNMT1 for penetrating into hemimethylated DNA and provokes rapid DNA hypomethylation and robust transcriptional activation. Remarkably, GSK3685032 has improved in vivo tolerability compared with decitabine, showing greater target engagement and consequently higher and more durable hypomethylating activity. These translate into higher anticancer activity with complete tumor regression and enhanced OS in mouse preclinical models of AML.[168] The significant improvements compared with cytidine analogues provide enhanced clinical opportunities and could translate into higher efficacy in solid tumors. Future studies to define the clinical benefits of nonnucleoside DNMTi agents are expected.

Histone deacetylase inhibitors

The epigenomic landscape is shaped by the interplay between DNA methylation and histone modifications. Of the latter, acetylation status of histones is a crucial player in the fine-tuning of gene expression by modulating chromatin conformation and consequently the accessibility of transcription factors, co-activators, and co-repressors. Histone acetylation status is determined by the opposing actions of histone acetyl transferases (HATs) and histone deacetylases (HDACs), that add or remove acetyl groups to lysine residues in the histone tails, respectively (Figure 1). The HDAC family is divided into four classes of enzymes. Three of these are zinc-dependent proteins, while the members of class III (sirtuins) use nicotinamide adenine dinucleotide (NAD+) as a cofactor.[12] By interacting with the zinc-containing catalytic site of HDACs, HDAC inhibitors block substrate access and prevent lysine deacetylation, allowing a hyperacetylated state of the histone tails that promotes the more relaxed chromatin structure required for transcriptional activation (Figures 4A and 5). Importantly, HDACs also have multiple nonhistone substrates, so the impact of HDAC inhibition is extended to other proteins, including several transcription factors.

Vorinostat and romidepsin for the treatment of cutaneous T-cell lymphoma

Among first-generation HDACi agents, vorinostat (suberoylanilide hydroxamic acid-SAHA; Zolinza; Merck & Company) was granted FDA approval for the treatment of cutaneous T-cell lymphoma in

2006 supported by two CTs that demonstrated ORRs of 30% (95% CI, 19.7%–41.5%) and 31% (95% CI, 9.1%–61.4%).[141] Similar response rates of 34% (95% CI, 23%–46%; $n = 24$)[142] and 34% (95% CI, 25%–45%; $n = 33$)[143] led to the approval of the HDACi romidepsin (Istodax; Celgene Corporation) for the treatment of cutaneous T-cell lymphoma treatment in 2009. Both indications are included in the NCCN Guidelines for Primary Cutaneous Lymphomas (version 2.2022). In 2011, results from a CT in patients with R/R peripheral T-cell lymphoma (PTCL) showed an ORR of 25% (33 of 130 patients)[144] supported the accelerated FDA approval of romidepsin for the treatment of R/R PTCL. However, this approval recently was withdrawn because the subsequent confirmatory phase 3 study did not meet the primary efficacy end point[145] (Table 4).

Ongoing combination trials in hematologic and solid malignancies

As previously described for DNMTi agents, synergistic effects of HDACi agents and other anticancer drugs observed in preclinical studies have promoted the rational design of combination trials (Figure 4B). Several studies have shown the potential of HDACi agents as priming modulators of immunotherapy by increasing PD-L1 expression and reducing regulatory T cells (Figure 5).[169,170] In breast cancer mouse models, vorinostat in combination with PD-1 and CTLA-4 blockage promoted T-cell tumor infiltration and tumor regression and increased survival.[169] Dual combination of vorinostat with the anti-PD1 agent pembrolizumab is in a phase 1 trial in advanced prostate, renal, or urothelial tumors (ClinicalTrials.gov identifier NCT02619253) and in DLBCL, follicular lymphoma (FL), or Hodgkin lymphoma (ClinicalTrials.gov identifier NCT03150329); in a phase 1/2 trial in NSCLC (ClinicalTrials.gov identifier NCT02638090); and in a phase 2 trial in squamous cell carcinoma (ClinicalTrials.gov identifier NCT04357873) (Table 4).

Preclinical studies have also shown the capacity of vorinostat to reverse hormone therapy resistance in an ER-positive breast cancer murine model by redirecting cells into apoptosis,[171] hence the combination of vorinostat with antiestrogen drugs to maximize the clinical benefits of hormone therapies has been assessed in CTs. A phase 2 study combining vorinostat with tamoxifen in patients with hormone therapy-resistant breast cancer patients (ClinicalTrials.gov identifier NCT00365599) obtained an ORR of 19% (8 of 43 patients), a clinical benefits rate (ORR and stable disease >24 weeks) of 40%, and a median OS of 29 months (95% CI, 20–38.5 months).[172] Remarkably, correlative results from that study identified HDAC2 expression as a predictive marker and histone hyperacetylation as a suitable pharmacodynamic marker for the efficacy of this combination.[172] A triple combination, adding immune checkpoint blockage, has also been evaluated. In this regard, although the randomized phase 2 trial combining vorinostat with tamoxifen and pembrolizumab (ClinicalTrials.gov identifier NCT02395627) has been prematurely stopped because of insufficient efficacy in an unselected patient population, it has helped define an exhausted T-cell immune signature in patients with PD-L1–negative, ER-positive breast cancer who are more likely to benefit from this treatment.[173] The use of this immune signature to preselect likely responders could maximize the benefits of this triple combination. Importantly, an observed HDACi-dependent decrease in regulatory T cells contributed to the efficacy observed in responding patients, supporting the role of HDACi agents as modulators of the immune response.[173]

The disruption of DNA damage sensing and repair processes by HDACi agents also promoted their use in tumor radiosensitization,[174,175] although studies showing clinical benefits are limited to a few examples. A phase 2 trial in patients with neuroblastoma comparing ^{131}I-metaiodobenzylguanidine (MIBG) radiotherapy alone or in combination with vorinostat or vincristine and irinotecan, determined that MIBG plus vorinostat had the highest ORR (32%; 95% CI, 18%–51%) after the first course compared with 14% on either of the other two arms, with manageable toxicity (ClinicalTrials.gov identifier NCT02035137).[176] Also, in a phase 1 study combining vorinostat with radiotherapy as neoadjuvant treatment in pancreatic cancer after chemotherapy, the regimen was well tolerated, and antitumor activity was observed (ClinicalTrials.gov identifier NCT02349867),[177] warranting further investigation in this aggressive tumor type.

Belinostat and tucidinostat for the treatment of peripheral T-cell lymphoma and other tumors

Turning now to the second-generation HDACi agents, belinostat was granted accelerated FDA approval for the treatment of R/R PTCL in 2014, after results from a single-arm trial in 120 patients demonstrated an ORR of 25.8% (95% CI, 18.3%–34.6%), including CR and PR rates of 10.8% (95% CI, 5.9%–17.8%) and 15.0% (95% CI, 9.1%–22.7%), respectively.[146] In 2015, accelerated approval was also granted to panobinostat, in combination with bortezomib and dexamethasone, for the treatment of patients with R/R multiple myeloma based on superior progression-free survival (PFS) in the panobinostat combination group ($n = 387$) compared with the placebo combination group ($n = 381$; PFS, 11.99 months [95% CI, 10.33–12.94 months] vs. 8.08 months [95% CI, 7.56–9.23 months]; HR, 0.63 [95% CI, 0.52–0.76]; $p < .0001$) reported in the phase 3 PANORAMA1 study (ClinicalTrials.gov identifier NCT01023308).[147] However, this approval recently was withdrawn in the United States because it was impossible to complete the necessary post-approval clinical research required for the FDA under the accelerated approval regulations for verifying the described clinical benefit (Table 4).

In 2014, the HDACi chidamide (tucidinostat) was approved in China for the treatment of R/R PTCL based on results from the phase 2 study ChiCTR-TNC-10000811 showing an ORR of 28% (22 of 79 patients), a PFS of 2.1 months (range, from 1 day to 44.9 months), and an OS of 21.4 months (range, 0.3–50.1 months).[148] A second

indication was also approved in China in 2019 for the combination of chidamide with an aromatase inhibitor in postmenopausal patients with hormone receptor-positive, HER2-negative advanced breast cancer. Chidamide in combination with exemestane showed PFS benefit, from 3.8 months (95% CI, 3.7–5.5 months) in the placebo group to 7.4 months (95% CI, 5.5–9.2 months) in the chidamide group (HR, 0.75; 95% CI, 0.58–0.98; $p = .033$) and demonstrated manageable adverse effects in a phase 3 trial (ClinicalTrials.gov identifier NCT02482753[149]; Table 4).

Combination trials with entinostat in hematologic and solid malignancies

Additional HDACi agents have been developed to improve the selectivity against HDAC family members, with the objective of reducing the toxicity that limited the potential of first-generation and second-generation HDACi agents. Among these agents, the benzamide derivative entinostat is the one with the greatest number of open CTs. Entinostat is a potent and selective inhibitor of class I and IV HDACs that received FDA Breakthrough Therapy Designation status in 2013 for the management of ER-positive advanced breast cancer in combination with exemestane based on data from the phase 2 ENCORE 301 study (ClinicalTrials.gov identifier NCT00676663). That trial showed an improved median OS, from 19.8 months in the exemestane only arm to 28.1 months in the combination arm (HR, 0.59; 95% CI, 0.36–0.97; $p = .036$).[150] Moreover, protein lysine acetylation in peripheral blood mononuclear cells was associated with prolonged PFS in the combination arm.[150] However, the long-awaited results of the E2112 phase 3 trial (ClinicalTrials.gov identifier NCT02115282) did not produce any better survival in the entinostat plus exemestane arm in patients who had aromatase inhibitor-resistant, hormone receptor-positive advanced breast cancer[151] (Table 4). Combined epigenetic therapy with low-dose azacitidine and entinostat has also been evaluated in extensively pretreated patients with recurrent metastatic NSCLC and advanced breast cancer. Results from the phase 1/2 trial in NSCLC (ClinicalTrials.gov identifier NCT00387465) showed a median survival in the entire cohort of 6.4 months (95% CI, 3.8–9.2 months), including a CR and a PR in a patient who remained alive and without disease progression 2 years after completing the epigenetic therapy.[178] In the breast cancer trial (ClinicalTrials.gov identifier NCT01349959), although no responses were seen in the triple-negative breast cancer (TNBC) cohort, one PR in a patient with hormone-resistant disease was observed, suggesting that a subset of women with hormone-resistant breast cancer may benefit from epigenetic therapy and/or the reintroduction of endocrine therapy with epigenetic therapy beyond progression.[179]

With the intention of exploiting the immune-enhancing effects of entinostat, several ongoing CTs are also testing combinations of this HDACi with immunotherapeutic agents in NSCLC, melanoma, and CRC (phase 1b/2; ENCORE-601; ClinicalTrials.gov identifier NCT02437136), metastatic renal cell carcinoma (phase 2; ClinicalTrials.gov identifier NCT03552380), melanoma (phase 2; ClinicalTrials.gov identifier NCT03765229), bladder cancer (phase 2; ClinicalTrials.gov identifier NCT03978624), cholangiocarcinoma and pancreatic cancer (phase 2; ClinicalTrials.gov identifier NCT03250273), lymphoma (phase 2; ClinicalTrials.gov identifier NCT03179930), MDS (phase 1; ClinicalTrials.gov identifier NCT02936752), and several advanced solid tumors (phase 2; ClinicalTrials.gov identifier NCT02909452) (Table 4). Additional HDACi agents in CTs are listed in Figure 4A.

Proteolysis-targeting chimeras to degrade HDACs

Despite the improvements in second-generation HDACi agents, their relatively low efficacy in monotherapy regimens and the side effects associated with the lack of isoform specificity have reinforced the need to develop new strategies. An emerging approach is the use of proteolysis-targeting chimeras (PROTACs), a technology that hijacks the intracellular ubiquitin proteasome system to regulate protein function by degrading target proteins instead of inhibiting them. PROTACs are heterobifunctional molecules composed of a ligand for binding target protein, a linker, and a ligand for recruiting E3 ligase. The simultaneous binding to the target protein and an E3 ligase promotes the ubiquitination and subsequent proteasomal degradation of the target protein (Figure 4C). Moreover, because PROTAC-induced degradation is a catalytic process, PROTACs could act at very low doses, which is a valuable advantage in the clinical setting. In addition, PROTAC-mediated degradation is not a competitively driven event, as are traditional inhibitors, and thus is less susceptible to mutations and increases in target expression, which enables it to overcome potential resistance mechanisms.[180] This strategy has recently been used to design HDAC6-targeting PROTACs.[181–183] Remarkably, promising antiproliferation activity in multiple myeloma cells has been described for HDAC6 degraders that attach the E3 ligase ligand pomalidomide to the HDAC6 selective inhibitor Nexturastat A.[183] Deregulation of HDAC6 is related not only to cancer but also to other diseases, such as neurodegenerative disorders and pathologic autoimmune responses,[184] thus expanding the therapeutic potential of HDAC6-targeting PROTACs. Further research in the field is warranted to evaluate the clinical opportunities of this strategy for modulating epigenetic enzymes.

Histone methyltransferase inhibitors

In addition to broad epigenetic reprogrammers, such as DNMTi and HDACi agents, the spectrum of epidrugs has been extended to a more specific targeted therapy based on the presence of activating mutations of epigenome-modifying enzymes, such as the use of tazemetostat (EPZ-6438, E7438) for patients harboring mutations in *EZH2*.[185]

EZH2, the catalytic subunit of PRC2, mediates transcriptional silencing through trimethylation of histone H3 on lysine 27

(H3K27me3; Figures 4A and 5). EZH2 is overexpressed in several tumor types, including melanoma and breast, bladder, endometrial, renal cell, liver, and lung cancers, leading to increases in H3K27me3 and concomitant repression of tumor suppressor genes. The correlation of high levels of EZH2 with a poor prognosis and tumor aggressiveness in several tumor types aroused initial interest in EZH2 as an antitumor target, but the identification of activating mutations in approximately 20% of germinal center DLBCL cases and in 10%–25% of FL cases[186] boosted the interest in EZH2 inhibitors (EZH2i), giving rise to a new opportunity for precision medicine.[130]

Tazemetostat for the treatment of follicular lymphoma and epithelioid sarcoma

The first-in-class EZH2i tazemetostat was granted FDA approval in 2020 for R/R FL after the evidential support of a phase 2 trial (ClinicalTrials.gov identifier NCT01897571) showing an ORR of 69% (95% CI, 53%–82%; 31 of 45 patients) in the *EZH2*-mutated cohort versus 35% (95% CI, 23%–49%; 19 of 54 patients) in the *EZH2* wild-type cohort.[152] Tazemestostat also received accelerated FDA approval in 2020 for the treatment of epithelioid sarcoma, a rare and aggressive soft tissue sarcoma subtype that shows EZH2-oncogenic dependence. The loss of INI1/SMARCB1, a component of the SWI/SNF chromatin-remodeler complex, is a molecular hallmark of epithelioid sarcoma that leads to the constitutive and oncogenic activation of EZH2.[187] The approval of tazemetostat in patients with INI1-deficient epithelioid sarcoma was based on a phase 2 trial (ClinicalTrials.gov identifier NCT02601950) that showed an ORR of 15% (95% CI, 7%–26%; 9 of 62 patients).[153] A phase 3 trial of tazemetostat plus doxorubicin in the front-line setting in epithelioid sarcoma is currently underway (ClinicalTrials.gov identifier NCT04204941) (Table 4).

Valemetostat for the treatment of adult T-cell leukemia/lymphoma

The greater activity of dual inhibitors for EZH1 and EZH2 to reduce cellular H3K27me3 and their superior antitumor efficacy in murine models of hematologic malignancies compared with selective EZH2 inhibition[188] led to the evaluation of the clinical efficacy. The positive results from a phase 2 trial of valemetostat in adult T-cell leukemia/lymphoma, with an ORR of 48% (90% CI, 30.5%–65.9%; 12 of 25 patients), including five CRs and seven PRs (ClinicalTrials.gov identifier NCT04102150),[154] supported the recent acceptance of valemetostat by the Japanese Ministry of Health, Labor, and Welfare as the first dual EZH1/2 inhibitor approved for the treatment of R/R adult T-cell leukemia/lymphoma (Table 4). In addition, the combination of valemetostat with ipilimumab is under study in a phase 1 trial for the treatment of patients with metastatic prostate, urothelial, or renal cell cancer (ClinicalTrials.gov identifier NCT04388852).

Combination trials with histone methyltransferase inhibitors for the treatment of hematologic and solid malignancies

Preclinical studies showed that Bap1 loss in mice results in increased Ezh2 expression and H3K27me3 levels and, more significant still, that *BAP1*-mutant mesothelioma cells are sensitive to EZH2 pharmacologic inhibition.[189] These findings prompted clinical investigations of tazemetostat in malignant mesothelioma with BAP1 inactivation. However, limited clinical benefits were observed, with an ORR of only 3% ($n = 2$), although disease stabilization was observed in 64% of patients ($n = 47$; ClinicalTrials.gov identifier NCT02860286).[190] The combination of tazemetostat with immune checkpoint blockage (Figure 4B), pembrolizumab for head and neck squamous cell carcinoma (phase 1/2; ClinicalTrials.gov identifier NCT04624113) and urothelial tumors (phase 1/2; ClinicalTrials.gov identifier NCT03854474), and durvalumab for multiple solid tumors (phase 2; ClinicalTrials.gov identifier NCT04705818) are also undergoing CTs (Table 4).

DOT1-like histone lysine methyltransferase (DOT1L), the only identified H3K79 methyltransferase, has also been a target for cancer treatment, particularly in acute leukemias involving *MLL* gene rearrangements because chimeric MLL proteins recruit DOT1L to aberrant target sites, promoting the ectopic expression of genes such as *HOXA9* and *MEIS1*.[191] Although early CTs (ClinicalTrials.gov identifiers NCT02141828 and NCT03701295) have noted only modest clinical efficacy, preclinical studies demonstrating that the DOT1L inhibitor pinometostat sensitizes pediatric AML cells to treatment with the multikinase inhibitor sorafenib, irrespective of *MLL* rearrangements,[192] could lead to novel therapeutic strategies for pediatric patients with AML.

Histone demethylase inhibitors

Dysregulation and overexpression of the lysine-specific histone demethylase LSD1 (also known as KDM1A) have been observed in various hematopoietic malignancies and solid tumors, including cancers of the breast, lung, colorectum, and prostate, where they have been linked to aggressiveness and a poor prognosis.[193–196] LSD1 acts mainly as a transcriptional corepressor by demethylating H3K4me1/2, a histone mark linked to active transcription.[197] However, in association with the androgen receptor (AR), LSD1 enzymatic specificity is altered to the repressive histone mark H3K9me1/2, leading to the derepression of AR target genes.[198] The identification of the role of LSD1 as a regulator of the balance between self-renewal and differentiation of stem cells, not only under physiologic conditions (hematopoietic and neuronal) but also in pathologic settings (cancer stem-like cells), presents attractive therapeutic opportunities. In this regard, it has been demonstrated that epigenetic modulation by inhibiting LSD1 provokes cellular reprogramming in tumor-initiating cells that mitigate cancer stemness through a distinctive molecular mechanism (Figure 5).[199–201]

LSD1 inhibitor-dependent differentiation and growth inhibition reported in preclinical studies promoted the initiation of several CTs to assess, for instance, pulrodemstat (CC-90011), iadademstat (ORY-1001), seclidemstat, and GSK2879552 (Figure 4A). Although results from phase 1 trials for GSK2879552 in AML (ClinicalTrials.gov identifier NCT02177812) and in small cell lung cancer (SCLC; ClinicalTrials.gov identifier NCT02034123) were unfavorable, and the risk-to-benefit ratio did not favor continuation of the studies, results with other inhibitors in monotherapy and/or combination are promising. A phase 1 trial testing pulrodemstat for the treatment of R/R non-Hodgkin lymphoma and solid tumors (ClinicalTrials.gov identifier NCT02875223) showed promising antitumor activity, particularly in patients with neuroendocrine neoplasms. Eight of 27 patients (30%) who had neuroendocrine tumors had stable disease for >6 months. Moreover, the only enrolled patient with R/R non-Hodgkin lymphoma had a CR, a result that warrants further studies.[202] The antitumor efficacy of CC-90011 in patient-derived xenograft SCLC models[203] has also promoted CTs in this cancer type. A phase 1 trial in patients with first-line, extensive-stage SCLC to explore CC-90011 in combination with cisplatin, etoposide, and/or carboplatin with or without nivolumab is ongoing (ClinicalTrials.gov identifier NCT03850067). Iadademstat has shown a good safety profile and evidence of clinical activity as a single agent, including a patient who had CR, in a phase 1 trial among patients with R/R AML (EudraCT No. 2013-002447-29).[155] Moreover, the combination of iadademstat with azacitidine for the treatment of AML produced an ORR of 73% (five CRs, six CRs with incomplete hematologic recovery, and five PRs) in preliminary results from the ALICE Trial (EudraCT no. 2018-000482-36).[156] Seclidemstat also demonstrated activity among patients with advanced sarcoma and had a manageable safety profile in a phase 1 trial (ClinicalTrials.gov identifier NCT03895684[204]) (Table 4).

LSD1 inhibitors for the treatment of Ewing sarcoma and combination trials in other cancer types

LSD1 inhibitors are also a promising therapy for Ewing sarcoma because LSD1 is a critical functional partner for EWS/FLI, the driver fusion protein in Ewing sarcoma that arises from the characteristic t (11;22) translocation. EWS/FLI alters the function of LSD1-containing complexes through two different mechanisms: (1) direct recruitment of NuRD-LSD1 repressor complexes that lead to transcriptional inhibition[205]; and (2) inducing dynamic, genome-wide reorganization of LSD1 that reshapes the enhancer landscape in Ewing sarcoma cells, resulting in the activation of different target genes.[206] Seclidemstat, in monotherapy or in combination with topotecan and cyclophosphamide, is currently under phase 1/2 study in patients with R/R Ewing sarcoma or Ewing-related sarcomas who have FET-family translocations (ClinicalTrials.gov identifier NCT03600649). Furthermore, a rollover protocol to allow continued access to seclidemstat for patients who are still receiving clinical benefit on completed or closed seclidemstat studies is underway (ClinicalTrials.gov identifier NCT05266196).

Several studies have demonstrated that the demethylase function of LSD1 is not restricted to histones. For example, a numerous nonhistone proteins have been detected as targets of LSD1 activity.[207] The DNA methyltransferase DNMT1 is one of these nonhistone targets. LSD1-dependent demethylation stabilizes DNMT1 and is required for the maintenance of global DNA methylation.[208] At least three phase 1/2 CTs to test the combination of LSD1 inhibitors and DNMT inhibitors for the treatment of hematologic malignancies are taking place. The first trial is studying the pharmacologic inhibition of LSD1 with seclidemstat in combination with azacytidine for chronic myelomonocytic leukemia and MDS (ClinicalTrials.gov identifier NCT04734990). The second is testing the triple combination of the LSD1 inhibitor CC-90011, azacytidine, and venetoclax in patients with AML (ClinicalTrials.gov identifier NCT04748848). The third trial is testing the combination of iadademstat with azacytidine in older patients with AML as first-line therapy (EudraCT No. 2018-000482-36). In addition, preclinical data showing that LSD1 inhibition improves the therapeutic efficacy of immune checkpoint blockade by enhancing tumor immunogenicity and T-cell infiltration[209–211] have led to the initiation of CTs to test combinations aimed at maximizing the benefits of immunotherapy in tumor types that have limited responses, such as SCLC. A phase 2 study to test the efficacy of CC-90011 plus nivolumab in advanced SCLC or squamous NSCLC is underway (ClinicalTrials.gov identifier NCT04350463), and a phase 1/2 trial to test bomedemstat (IMG-7289) plus atezolizumab in newly diagnosed, extensive-stage SCLC has recently started to enroll patients (ClinicalTrials.gov identifier NCT05191797).

Computational chemistry approaches have also been used to design LSD1-HDAC dual inhibitors. A successful example is JBI-802, a first-in-class potent and selective dual inhibitor of LSD1 and HDAC6/8. Synergistic antitumor activity demonstrated in animal models[212] promoted a phase 1/2 CT for patients with advanced solid tumors that recently was opened (ClinicalTrials.gov identifier NCT05268666).

Bromodomain and extraterminal domain protein inhibitors

The role of bromodomain and extraterminal domain (BET) proteins as chromatin regulators makes them attractive targets for cancer therapy. The BET family of chromatin *readers* (BRD2, BRD3, BRD4, and BRDT) contains a bromodomain that recognizes acetylated lysines, triggering chromatin remodeling and transcriptional activation through the recruitment of other proteins. BET proteins act as key regulators of oncogene expression by controlling super enhancers that regulate critical oncogenic drivers, including *MYC*,[213] but a broader function as master regulators of global transcription elongation has also been described.[214]

BET inhibitors for the treatment of NUT midline carcinoma and other tumors

Oncogenic roles of BET proteins were first revealed in NUT midline carcinoma (NMC), poorly differentiated, highly aggressive tumors whose genetic hallmark is the fusion of NUT (NUTM1; nuclear protein in testis) mainly with BRD4 (70% of cases)[215] or BRD3 (6% of cases).[216] BET inhibitors (Figure 4A) have been tested in phase 1 trials for the treatment of NMC and other solid tumors. The trial with the BET inhibitor (BETi) birabresib (MK-8628) was prematurely terminated because of limited efficacy (ClinicalTrials.gov identifier NCT02698176), but results for molibresib (GSK525762) from the trial (ClinicalTrials.gov identifier NCT01587703) are promising. Of the 19 patients who had NMC, four achieved a confirmed or unconfirmed PR, eight had stable disease as their best response, and four had a PFS for >6 months.[217] Moreover, a phase 1/2 trial has recently been opened to assess the addition of the BETi ZEN-3694 to chemotherapy (etoposide and cisplatin) for NMC treatment (ClinicalTrials.gov identifier NCT05019716).

The first small-molecule BETi agents, such as JQ1, were key to demonstrating the oncogenic activity of BET proteins and the impact of BET inhibition on the expression of several oncogenes. This effect on critical oncogenes could drive the antitumorigenic activity of BETi agents (Figure 5) observed in preclinical models. However, their poor pharmacokinetic profile, short half-life, and low oral bioavailability limited the clinical applications. TEN-010 (RO6870810), which is similar in structure to JQ1, with improvements in pharmacologic properties, entered into CTs for the treatment of AML and MDS (phase 1; ClinicalTrials.gov identifier NCT02308761) and solid tumors (phase 1; ClinicalTrials.gov identifier NCT01987362). A lack of efficacy of TEN-010 in monotherapy for AML and MDS has recently been reported,[218] but evidence of target engagement and preliminary single-agent activity in NMC, solid tumors, and DLBCL with MYC deregulation supports further exploration, particularly in MYC-driven cancers.[219] Moreover, TEN-010 in combination with venetoclax (a BCL-2 inhibitor) and rituximab (anti-CD20) in patients with DLBCL (phase 1; ClinicalTrials.gov identifier NCT03255096) has shown encouraging results, with an ORR of 38.5% (15 of 39 patients) and a CR rate of 20.5% (8 of 39 patients), suggesting further studies.[220] The combination of TEN-010 with atezolizumab in TNBC and ovarian cancer also was included in a CT (ClinicalTrials.gov identifier NCT03292172), but the study was terminated because of portfolio prioritization.

Combinations of other BETi agents with immunotherapies are also being tested (Figure 4B). For instance, ZEN-3694 with nivolumab is in a phase 1 trial for the treatment of solid tumors (ClinicalTrials.gov identifier NCT04840589); and the triple combination ZEN-3694 plus pembrolizumab and the antiandrogen enzalutamide is in a phase 2 trial for the treatment of metastatic, castration-resistant PCa (ClinicalTrials.gov identifier NCT04471974). The efficacy of BMS-986158 in monotherapy or in combination with nivolumab is also under study for selected advanced cancers (phase 1/2; ClinicalTrials.gov identifier NCT02419417). Moreover, studies showing that BETi agents avoid *kinase reprogramming* by preventing the transcription of alternative tyrosine kinase receptors and proteins that drive acquired resistance have demonstrated the value of using them to overcome resistance to receptor tyrosine kinase inhibitors and tyrosine kinase inhibitors.[221,222] In TNBC cells treated with lapatinib, BET inhibition using JQ1 or I-BET151 suppresses transcription of many lapatinib-induced kinases involved in resistance, generating a durable response.[221] An analogous effect has been described for the next-generation BETi PLX51107 in *BRAF*-mutant melanoma models treated with BRAF/MEK inhibitors (BRAFi/MEKi). PLX51107 suppresses adaptive receptor tyrosine kinase upregulation in response to targeted therapy; and, although the triple combination BRAFi/MEKi/BETi is highly toxic, intermittent BETi combined with continuous BRAFi/MEKi treatment was tolerable and improved durable tumor inhibition outcomes.[223] Based on these lines of evidence, a phase 1 trial to test a combination of the BETi ZEN-3694 with the MEKi binimetinib in patients with solid tumors harboring RAS pathway alterations or TNBC has recently been launched (ClinicalTrials.gov identifier NCT05111561).

Another promising combination is based on the ability of BET inhibition to impair the transcription of several key proteins involved in homologous recombination-mediated DNA damage repair.[224–226] Hence, combination with a BETi could be used to sensitize homologous recombination-proficient cancers to poly-(ADP-ribose) polymerase (PARP) inhibitors (PARPi) and to resensitize acquired PARPi resistance. The strong synergy between PARP inhibition and BET inhibition observed in homologous recombination-proficient breast and ovarian preclinical models[224,227] has prompted the assessment of the clinical benefits in patients. A phase 2 trial to test the efficacy of ZEN-3694 and talazoparib in ovarian cancer patients who progressed on prior PARPi therapy has begun (ClinicalTrials.gov identifier NCT05071937).

Degraders to target BET proteins

The development of degraders to target BET proteins, including PROTACs and molecular glues (Figure 4C), is also a matter of active research. Among the molecules based on PROTAC technology, the pan-BET degrader ARV-771 has shown antitumor potential in castration-resistant PCa mouse models, in which it suppresses both AR signaling and AR levels[228]; and treatment with the QCA570 degrader led to complete and durable tumor regression in acute leukemia xenograft models.[229] Another example, the dBET6 degrader, was key to defining the role of BET proteins as master regulators of transcription elongation. Mechanistically, degraders elicit a transcriptional response characterized by the global disruption of productive transcription elongation, in contrast to the preferential displacement from super enhancers and downregulation of a discrete set of super enhancer–regulated genes achieved by BET inhibition. Moreover, dBET6 led to improved survival in a disseminated mouse model of T-cell acute lymphoblastic leukemia compared with JQ1.[214] In the case of molecular glue-degraders, efforts have

focused on BRD9 after the discovery of critical functional BRD9-dependency in synovial sarcoma, a deadly cancer with limited treatment options that primarily affects adolescents and young adults. The FHD-609 degrader is the subject of a phase 1 trial (ClinicalTrials.gov identifier NCT04965753) for the treatment of synovial sarcoma, and the CFT8634 degrader has recently received FDA clearance to proceed with a phase 1/2 CT in synovial sarcoma and SMARCB1-null solid tumors (ClinicalTrials.gov identifier NCT05355753).

Inhibitors targeting alterations that disrupt the epigenome

Gain-of-function mutations in isocitrate dehydrogenases *IDH1* and *IDH2*, which are frequent in lower grade gliomas but also occur in AML and other malignancies,[230–232] confer neomorphic enzyme activity, which causes further processing of α-ketoglutarate to 2-hydroxyglutarate (2HG).[233] 2HG acts as a competitive inhibitor of multiple α-ketoglutarate–dependent dioxygenases, including the Jumonji-C domain family of histone demethylases and the TET family of DNA demethylases. Consequent inhibition of TET catalytic activity in *IDH1*/*IDH2*-mutant cells disrupts the epigenome by altering the global methylation landscape, impairs cellular differentiation, and promotes cancer.[234–236] For these reasons, mutant IDH has become a very attractive therapeutic target, and several IDH inhibitors have been designed for the purpose of overcoming the action of 2HG.

Treatment of *IDH1*/*IDH2*-mutant tumors

Preclinical studies demonstrating the efficacy of enasidenib (AG-221; Idhifa; Bristol Myers Squibb) and its ability to suppress 2HG production and induce cellular differentiation created the possibility of starting CTs. Favorable results from a single-arm trial of patients with R/R AML who had *IDH2* mutations (ClinicalTrials.gov identifier NCT01915498), which reported an ORR of 40.3% (95% CI, 33%–48%; 71 of 176 patients), including a CR in 34 patients (19.3%; 95% CI, 13.8%–25.9%),[157] supported the FDA approval of this first IDH2-mutant inhibitor in 2017 (Table 4). Currently, there are almost 20 CTs enrolling mainly patients who have *IDH2*-mutated R/R AML with the aim of evaluating the efficacy of enasidenib in monotherapy (e.g., ClinicalTrials.gov identifiers NCT03744390 and NCT04203316) or in combination with the DNMT inhibitor azacitidine (ClinicalTrials.gov identifier NCT03683433), the BCL-2 inhibitor venetoclax (ClinicalTrials.gov identifier NCT04092179), and the JAK1/JAK2 inhibitor ruxolitinib (ClinicalTrials.gov identifier NCT04281498). There also are nine active, nonrecruiting trials.

The scenario is similar for the IDH1-mutant inhibitor ivosidenib (AG-120; Tibsovo; Agios Pharmaceuticals/Servier Pharmaceuticals LLC), which the FDA approved in 2018 for the treatment of adults with R/R AML harboring *IDH1* mutations, based on positive results from the AG120-C-001 trial (ClinicalTrials.gov identifier NCT02074839), which achieved an ORR of 41.6% (95% CI, 32.9%–50.8%; 52 of 125 patients), with CR or CR with partial hematologic recovery in 38 patients (30.4%, 95% CI, 22.5%–39.3%).[158] The FDA indication was extended to patients with newly diagnosed, *IDH1*-mutated AML in 2019, after the benefits shown in the AG120-C-001 trial, with CR and CR with partial hematologic recovery in 14 of 33 patients (42.4%, 95% CI, 25.5–60.8), including CR in 10 patients (30.3%; 95% CI, 15.6%–48.7%).[159] More recently, the use of ivosidenib in combination with azacitidine was FDA approved for newly diagnosed, *IDH1*-mutated AML, in accordance with favorable results from the AG120-C-009 trial (ClinicalTrials.gov identifier NCT03173248), which reported a significantly higher CR rate in the ivosidenib plus azacitidine arm compared with the placebo plus azacitidine arm (CR rate, 47% [95% CI, 35%–59%] vs. 15% [95% CI, 8%–25%]) and improved median OS (24.0 months [95% CI, 11.3–34.1 months] vs. 7.9 months [95% CI, 4.1–11.3 months]; HR, 0.44; 95% CI, 0.27–0.73; $p = .0010$).[135] All of these indications of ivosidenib for AML treatment are included in the NCCN Guidelines for AML (version 2.2022) (Table 4).

In 2021, ivosidenib was also FDA approved to treat advanced cholangiocarcinomas with *IDH1* mutation, after the ClarIDHy phase 3 CT (AG120-C-005; ClinicalTrials.gov identifier NCT02989857) demonstrated good tolerability and favorable PFS and OS benefit versus placebo (median PFS, 2.7 months [95% CI, 1.6–4.2 months] vs. 1.4 months [95% CI, 1.4–1.6 months]; HR, 0.37; 95% CI, 0.25–0.54; one-sided $p < .0001$; median OS, 10.3 months [95% CI, 7.8–12.4 months] vs. 7.5 months [95% CI, 4.8–11.1 months]; HR, 0.79; 95% CI, 0.56–1.12; one-sided $p = .09$).[160] The NCCN Guidelines include this application for the treatment of *IDH1*-mutant cholangiocarcinoma (NCCN Guidelines for Hepatobiliary Cancers, version 2.2022) and also include the use of ivosidenib as a treatment option for patients with *IDH1*-mutant conventional or dedifferentiated chondrosarcoma (NCCN Guidelines for Bone Cancer, version 1.2023). This last indication relies on results from the AG120-C-002 trial (ClinicalTrials.gov identifier NCT02073994), which evaluated the response to ivosidenib in 21 patients with advanced chondrosarcoma and reported a median PFS of 5.6 months (95% CI, 1.9–7.4 months), PFS at 6 months of 39.5%, and stable disease in 11 patients (52%).[161] In addition to several CTs currently open to assess the efficacy of ivosidenib as a single agent or in combination with azacitidine in *IDH1*-mutant hematologic malignancies, the combination of ivosidenib with nivolumab is being assessed in a phase 2 trial for *IDH1*-mutant, advanced solid tumors (ClinicalTrials.gov identifier NCT04056910), and a phase 1 trial of ivosidenib with mFOLFIRINOX (combined folinic acid, 5-flurouracil, irinotecan, and oxaliplatin) in patients with resectable pancreatic adenocarcinoma has recently started (ClinicalTrials.gov identifier NCT05209074) (Table 4).

Dual inhibitors of mutant *IDH1*/*IDH2* are also in CTs. Among them, vorasidenib (AG-881), the first-in-class, brain-penetrant dual inhibitor, has shown preliminary antitumor activity in patients with recurrent or progressive, nonenhancing, IDH-mutant, lower grade gliomas (ClinicalTrials.gov identifier NCT02481154).[237] It is also undergoing a phase 1 trial for patients with advanced solid tumors

who have *IDH1*/*IDH2* mutations, including gliomas (ClinicalTrials.gov identifier NCT02481154). HMPL-306 is another dual-mutant *IDH1*/*IDH2* inhibitor in a phase 1 trial for hematologic malignancies (ClinicalTrials.gov identifiers NCT04272957 and NCT04764474) and solid tumors (ClinicalTrials.gov identifier NCT04762602).

An additional strategy for targeting *IDH1* genetic alterations has been the development of vaccines because the most common *IDH1* mutation in gliomas, affecting the arginine residue Arg132, encodes a tumor-specific neoantigen: IDH1(R132H).[238] Results from the NOA-16 first-in-humans trial using an IDH1(R132H)-specific peptide vaccine noted an ORR of 84.4% (95% CI, 67.21%–94.72%; 27 of 32 patients). More importantly, in that trial, the 2-year and 3-year PFS rates were 82% (95% CI, 62.3%–92.1%) and 63% (95% CI, 44%–77%), respectively (ClinicalTrials.gov identifier NCT02454634).[239] A current phase 1 trial is testing the combination of an IDH1(R132H)-specific peptide vaccine with the anti–PD-L1 avelumab in progressive diffuse glioma (ClinicalTrials.gov identifier NCT03893903). Another two vaccines, PEPIDH1M and IDH1R132H-DC, are also in phase 1 trials in IDH-mutant gliomas in combination with chemotherapy (ClinicalTrials.gov identifiers NCT02193347 and NCT02771301).

FUTURE DEVELOPMENTS

Human tumors are intricate ecosystems composed of diverse cells, including malignant, stromal, and immune populations, whose precise characterization is masked in bulk analyses of tumor tissues. Intratumoral heterogeneity is one of the greatest challenges facing clinical oncology because different cell populations could have different sensitivity to treatment, proliferation rates, and/or metastatic potential; thus preexisting nontarget populations could persist after treatment and result in treatment resistance or disease relapse. The emergence of single-cell technologies is providing novel opportunities to unravel the complexity of tumor heterogeneity at the highest resolution. Using this strategy, it is now possible to zoom into the cancer epigenome and explore the epigenetic components that regulate different aspects of tumor heterogeneity, including clonal heterogeneity, the tumor microenvironment (TME), spatial organization, and the intricate mechanisms of intratumoral differentiation, metastasis, and drug response.[240] For example, single-cell approaches could reveal specific gene-regulatory programs that give rise to resistance to epigenetic drugs and could be useful for identifying therapeutic vulnerabilities that can be tackled to improve the clinical benefits of these therapies. Moreover, characterization of the TME could guide the combined use of epidrugs with other strategies, such as immunotherapies. Single-cell approaches are also extraordinary strategies for characterizing MRDs, those rare residual cells that survive through treatments and ultimately underlie tumor recurrence. The identification of molecular signaling pathways that drive the emergence of a cell population conferring treatment resistance offers new candidate vulnerabilities that could be exploited for therapeutics.

Recently developed methodologies for studying DNA methylation at single-cell resolution, such as multiplexed single-cell reduced-representation bisulfite sequencing,[241,242] are unique tools with which to gain insights into epigenetic heterogeneity. Another single-cell approach that is revealing crucial information to better understand the dynamics of drug response is the transposase-accessible chromatin sequencing (ATAC-seq) assay at the single-cell level (scATAC-seq). By measuring DNA accessibility, this approach has demonstrated that cell states with distinct epigenomic profiles can respond differently to targeted therapy using a tyrosine kinase inhibitor.[243] The application of this technology has also revealed regulatory networks in malignant, stromal, and immune cells in the TME. For example, an analysis of scATAC-seq profiles from serial tumor biopsies before and after PD-1 blockade identified chromatin regulators of therapy-responsive T-cell subsets and revealed a shared regulatory program controlling T-cell exhaustion in basal cell carcinoma.[244] Moreover, by combining scATAC-seq with single-cell RNA sequencing, dynamic cell states modulated by epigenetic drugs could be defined. Therefore, considering, on the one hand, the effects of epigenetic drugs in reshaping the chromatin landscape and expression profiles and, on the other hand, the critical role of tumor heterogeneity in drug response, multiomic analysis at single-cell resolution will be key to deciphering and understanding the biologic effects of epidrugs in malignant cells and the TME and, consequently, to improving therapeutic strategies.

Many promising results demonstrating the synergy between epigenetic drugs and other anticancer therapies, as well as their potential to reverse acquired therapy resistance by modulating the sensitivity of cancer cells to other treatments (including immunotherapy, chemotherapy, radiation therapy, hormone therapy, and molecularly targeted therapy; Figures 4B and 5), are giving rise to new possibilities for maximizing the efficacy of cancer treatments. In-depth biologic knowledge generated with state-of-the-art technologies will guide the development of novel therapeutic strategies to extend the benefits of epidrugs to more patients with cancer by improving patient stratification or by the rational use of combination treatments.

Comprehensive characterization of the cancer epigenome using single-cell technologies will also broaden the portfolio of epigenetic biomarkers. For instance, the characterization of treatment-resistant clones at single-cell resolution will enable us to identify novel biomarkers that we can use to improve disease monitoring. Moreover, novel technologies to explore simultaneously several layers of the molecular information in individual cells will allow us to precisely elucidate the interplay between genetic and epigenetic mechanisms governing tumorigenesis. An example of this is single-cell chromatin overall omic-scale landscape sequencing (scCOOL-seq), which provides data about nucleosome positioning, chromatin accessibility, DNA methylation, copy number alterations, and ploidy in each individual cell.[245] A recent application of this technology, in combination with single-cell RNA sequencing, revealed two novel prognostic pancreatic ductal adenocarcinoma biomarkers (ZNF667 and ZNF667-AS1) that play crucial roles in suppressing the proliferation of pancreatic ductal adenocarcinoma cells.[246]

Today, epigenetics is at the cutting edge, and the field is expanding rapidly. Technological advances and investments made in research and development have enlarged the repertoire of epigenetic biomarkers and epidrugs. Moreover, cross-functional collaborations between multidisciplinary teams are facilitating the development of bench-to-bedside initiatives to improve the clinical management of patients with cancer using epigenetic approaches. The growth of applications in clinical oncology is clearing the path toward a new era of epigenetic health care solutions, fostering the expansion of the epigenetic market for the benefit of cancer patients.

CONFLICTS OF INTEREST

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