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Review

Advances in Epigenomic Sequencing and Their Applications in Cancer Diagnostics

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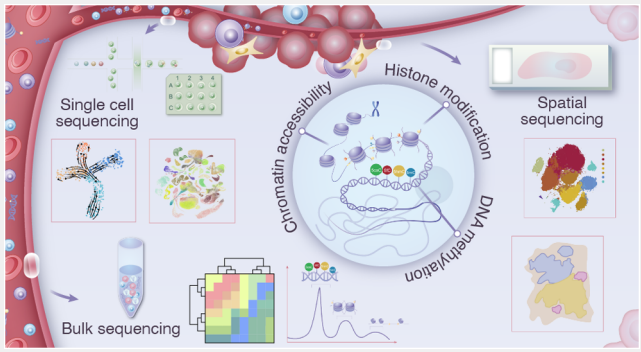
ABSTRACT: Cancer, a globally prevalent and life-threatening disease, remains a major area of focus in biomedical research. However, its substantial heterogeneity and complex pathogenesis continue to pose significant challenges for accurate diagnosis and effective treatment. The rapid advancement of epigenomic sequencing technologies has opened avenues by uncovering the epigenetic hallmarks and underlying pathology of cancer. As a result, these technologies have become invaluable tools in advancing cancer diagnostics and connecting research with clinical applications. This review briefly overviews epigenomic modifications and their significance in cancer diagnostics, highlighting potential epigenomic biomarkers with clinical applicability. We also examine emerging techniques in bulk and single-cell sequencing approaches, alongside spatial tools, highlighting their integration with multiomics technologies for cancer diagnostics. Particular attention is given to the analysis of key epigenetic characteristics, such as DNA methylation, histone modifications, and chromatin accessibility. Additionally, we summarize the diagnostic applications of these technologies and evaluate their current adoption in clinical settings. Challenges, limitations, and future directions for advancing epigenomic sequencing toward routine clinical diagnostics are also discussed. This review aims to provide scientists and clinicians with a comprehensive resource, encouraging further exploration and adoption of epigenomic sequencing technologies to drive progress in precision medicine.



KEYWORDS: *epigenomics, DNA methylation, chromatin accessibility, histone modifications, single-cell, spatial, sequencing, cancer diagnostics*

1. INTRODUCTION

Despite the advancement of various tools for early cancer diagnostics, the mortality rate of cancer remains high due to patient-specific variability and the intricate cellular microenvironment of cancer cells. The profile of the human genome has deciphered the hallmarks of many complex cancers. However, many symptoms may lack an identified associated gene or defined origin, yet they involve disrupted patterns of epigenetic modifications.[1,2] Epigenomics offers an additional layer of information within the genome, regulating its function and activity.[3] Epigenomics examines the distribution and roles of epigenetic modifications across the genome that are independent of the DNA sequence.[1,3] Such changes in the epigenome, including DNA methylation, histone modifications, chromatin accessibility, etc., are crucial for gene regulation and cancer biology.[4,5]

Epigenomic changes are among the earliest genomic alterations in cancer development.[6] A broader range of epigenetic factors, such as DNA methylation, has been identified as diagnostic markers and is being explored for early detection, predictive insights, and prognostic applications.[7,8] Numerous epigenomic biomarkers have been approved for clinical cancer diagnostics or registered for evaluation in clinical trials.[7,8] DNA methylation alterations are associated with abnormal histone modification patterns, resulting in adopting either condensed transcriptionally silent chromatin architectures or decondensed DNA-accessible conformations. Integrative capture and analysis of epigenomic modifications allow for the interpretation of patients' epigenetic profiles, providing valuable insights for cancer diagnostics.

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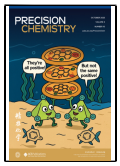

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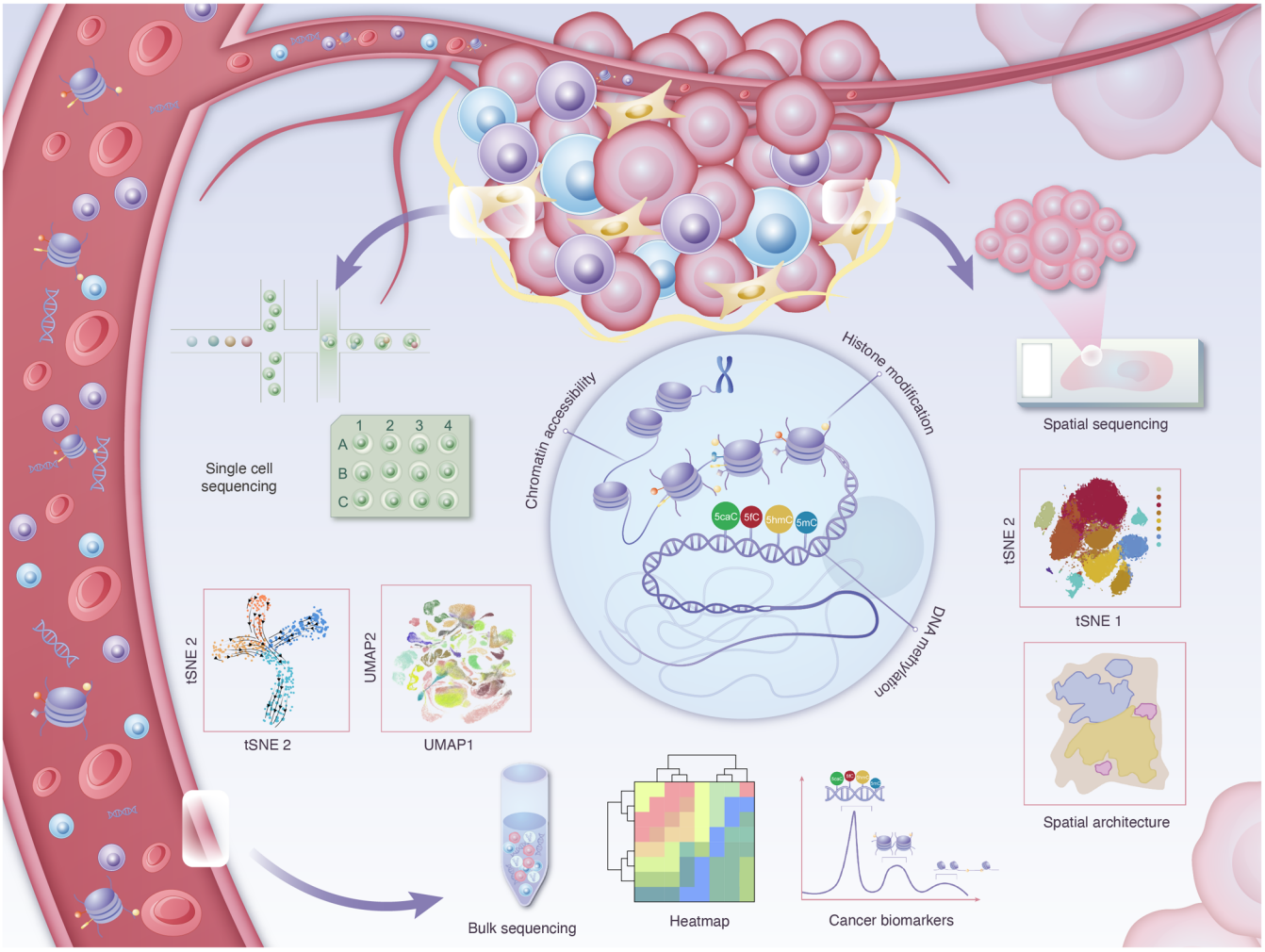

Figure 1. Epigenomic sequencing and multiomics analysis across bulk, single-cell, and spatiotemporal dimensions in cancer diagnostics.

Recent advancements in high-throughput techniques have deepened our insights into the epigenetic landscape of tumor development, exhibiting multiple malignancy-specific regulatory patterns and promising biomarkers for early detection, prognosis, and therapeutic monitoring. Epigenomic sequencing seeks to deliver an unbiased overview of the full spectrum of these modifications and enables large-scale assessment of epigenomic information in biospecimens, making it a key "omics" technology.[9] The primary goals of epigenomic sequencing include identifying and functionally annotating regulatory elements, analyzing cellular heterogeneity and the factors governing cell-type-specific functions, unraveling the dynamics of gene expression regulation under various physiological conditions, mapping developmental timelines, exploring heritable gene expression patterns and lineage determination, understanding disease mechanisms influenced by epigenomic factors, and discovering biomarkers for disease diagnosis and prognosis.[10]

A wide array of epigenomic sequencing technologies has been developed to analyze various 'omics' dimensions or layers of information, ranging from bulk and single-cell approaches to spatial tools and their integration with multiomics data.[10,11] Cancer diagnostics frequently utilize various biological samples to detect cancer indicators, track tumor progression, and evaluate treatment responses. These samples consist of various cell populations exhibiting diverse functional states. The common samples used in cancer diagnostics come in various formats, including body fluids (e.g., saliva, blood, cerebrospinal fluid, urine), tissue biopsies, and pathological sections.[12] Each of these sample types serves a specific purpose, ranging from early stage cancer detection to evaluating genetic mutations and metastasis. In Cancer Res. and clinical applications, epigenomic sequencing technologies offer distinct advantages when applied to specific sample types. For instance, bulk sequencing is particularly effective for analyzing methylation patterns in circulating cell-free DNA, making it a promising diagnostic tool for body fluids.[13] Single-cell technologies facilitate the detailed dissection of heterogeneity samples, enabling the extensive exploration and characterization of cell diversity.[11] Spatial sequencing methods can analyze genomic locations and other key features of biomarkers while preserving spatial context within pathological slides.[14] The newly emerged multiomics technologies facilitate integrative epigenomic profiling at single-cell or tissue-level resolution. Altogether, these technologies have revolutionized cancer diagnostics with transformative applications across various cancer types.

This review primarily highlights advancements in epigenomic sequencing technologies leveraging next-generation sequencing (NGS) and the utilizations in cancer diagnostics (Figure 1). We begin with a concise introduction to the biology of epigenomic modifications and provide a concise summary of potential and clinically available epigenomic

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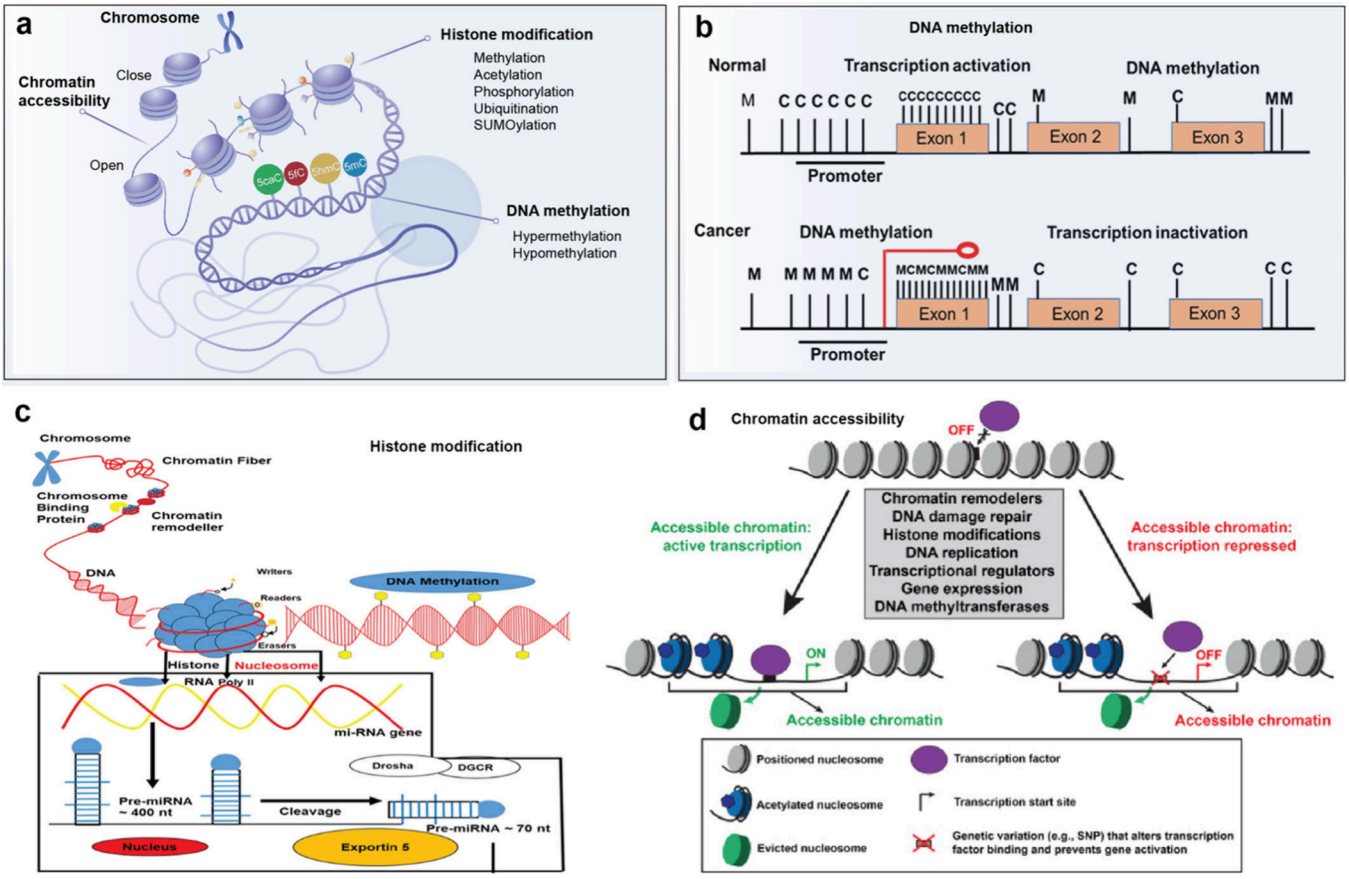

Figure 2. (a) Cancer epigenomes characterize abnormal DNA methylation, histone modifications, and chromatin openness that regulate transcription. (b) Altered DNA-methylation patterns in tumorigenesis. Reproduced from refs 17 and 80. Copyright 2022 Elsevier. (c) Dynamic regulation of epigenetic states through histone modifications. Reproduced from refs 17 and 80. Copyright 2022 Elsevier. (d) Chromatin accessibility maps reflect the topography of genome-wide chromatin dynamics. Reproduced from ref 88. Copyright 2019 Elsevier.

biomarkers for cancer diagnostics. We highlight emerging bulk, single-cell, and spatial epigenomics technologies, along with their integration into multiomics sequencing platforms, focusing on techniques for profiling DNA methylation, histone modifications, and chromatin accessibility. Then, we explore their applications in cancer diagnostics, including the detection of aberrant epigenomic patterns, tumor-specific mutations, cell lineage tracing, cell-specific atlas creation, and decoding of cellular spatial architectures for both fundamental research and medical translation. Finally, we discuss the challenges, limitations, and future directions for advancing these technologies toward clinical diagnostics.

2. EPIGENOMICS IN CANCER DIAGNOSIS

Dysregulated epigenetics, including aberrant DNA methylation, histone modifications, and chromatin remodeling, critically drive tumorigenesis and development in human cancer (Figure 2a). In this section, we mainly discuss the impact of epigenetic gene expression patterns and their associated phenotypes in cancer.

2.1. DNA Methylation

DNA methylation, a key epigenetic mechanism, has been widely investigated for its critical functions in gene regulation and maintaining genomic stability. In DNA methylation, DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group to cytosine's 5-position, primarily at CpG dinucleotides, resulting in the formation of 5-methylcytosine (5mC).[15] Alterations in DNA methylation include hypermethylation, hypomethylation, and the loss of imprinting.[16] In physiologically normal human cells, CpG islands, regions dense in CpG dinucleotides, typically remain unmethylated. (Figure 2b).[17] The unmethylated condition of CpG islands enables the binding of transcriptional activators, enhancing gene expression. In cancer cells, a common hypermethylation alteration is detected in CpG islands of tumor-suppressor genes, which induces transcriptional inactivation and the loss of their normal cellular functions. Upon methylation of a gene's promoter region, they inhibit the binding of transcription factors (TFs) necessary for gene activation.[18]

Methylation panels, consisting of specific genes with altered methylation patterns in cancer, have demonstrated potential for identifying various tumors, including breast, colorectal, lung, and pancreatic cancers.[19] DNA methylation-related gene expression changes are frequently observed, including hypermethylation-mediated transcriptional repression of tumor suppressor genes and hypomethylation activating repetitive elements.[20] DNA methylation profiling has uncovered tumor-specific hypermethylation patterns in CpG islands, facilitating the classification of tumor types and subtypes.[21,22] The distinct patterns of methylation sequences that exhibit coordinated gains or losses of CpG methylation can subclassify tumors into molecular subtypes,[23] such as gastric cancer,[24] colorectal cancer (CRC),[25] central nervous system tumors,[26] or squamous cell carcinoma.[27]

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Table 1. DNA Methylation-Based Detection of Clinical Application in Oncology

Markers	Cancer type	Sample type	Approve	Refs
GSTP1, APC, RASSF1	Prostate cancer	Tumor	_	40−42
NDRG4/BMP3 and KRAS mutation	Colorectal	Stool	FDA (2014)	43,44
			Asian Approve (NMPA, 2020)	
SEPT9	Colorectal (for HCC screening), liver	Blood	FDA (2016) European Certification (CE-IVD, 2011)	34,35,45,46
			Asian Approve (NMPA, 2014)	
			European Certification (CE-IVD, 2018)	
SHOX2	Lung cancer	EBUS-TBNA	European Certification (CE-IVD, 2010)	47
TWIST1, OTX1, ONECUT2 and FGFR3, TERT, HRAS mutation	Bladder cancer	Urine	_	48
BCAT1, IKZF1	Colorectal	Blood	_	49,50
BCAT1, CDO1, TRIM58, ZNF177	Lung cancer	Tumor tissue	_	51
RASSF1A, GSTP1, RARB	Breast cancer	Blood	_	38
p16/CDKN2A	Oral potential malignant disorder	Tumor tissue	_	52
SDC2	Colorectal	Stool	European Certification (CE-IVD, 2017), Asian Approve (MFDS, 2018; NMPA,2018)	53−55
SDC2/SFRP2	Colorectal	Stool	_	56
Methylation marker (28 target genes, 77GpC sites) and proteins markers	Liver	Blood	FDA BDD (2018); Asian Approve (NMPA accepted registration,2021)	57
ctDNA	Liver	Blood	FDA BDD (2019)	_
VASH2, CHFR, GRID2IP, CCNJ, F12	Liver	Blood	_	58
15 methylation markers	Bladder, urothelial	Urine	European Certification (CE-IVD, 2017)	59−61
ONECU2, VIM	Bladder, urothelial	Urine	FDA BDD (2021); European Certification (CE-IVD, 2020)	62
PENK	Bladder	Urine	_	63
SIM2, NKX1−1, TRNA-Cys	Bladder	Urine	_	64
CCNA1, VIM	Esophagus	Brush cell	FDA BDD (2020);	65
ZNF582	Esophagus	Oral cell	European Certification (CE-IVD, 2016)	66
SHOX2, PTGER4	Lung	Blood	European Certification (CE-IVD, 2017)	67
PAX1	Cervix	Cytology	European Certification (CE-IVD, 2016)	_
ZNF671, ASTN1, DLX1, ITGA4, RXFP3, SOX17	Cervix	Liquid- based cytology	European Certification (CE-IVD, 2019)	68,69
PITX2	Breast	Tumor	European Certification (CE-IVD, 2018)	70
MGMT	Glioblastoma	Tumor	European Certification (CE-IVD, 2015, 2016,2018)	71,72
Methylation markers	Cancer of unknow primary	Tumor	European Certification (CE-IVD, 2015)	73
ctDNA	>50 tumor types	Blood	European Certification (CE-IVD, 2022)	74−76
ctDNA	Colorectal, lung, liver, stomach, esophagus, breast, colon	Blood	_	77,78

DNA methylation markers offer several advantages over other biomarkers, including their stability, cost-effective amplification, and specificity to methylated DNA regions.[28] Aberrant methylation can often be detected prior to the appearance of visible cancer symptoms or during tumor progression.[6] Its widespread occurrence across specific tumor types makes DNA methylation an effective biomarker for early cancer detection. For example, genes like p16INK4a, MLH1, and RASSF1A show methylation has been detected in lung, colon, and breast cancers, etc.[29] Methylation alternation is also recognized as a diagnostic biomarker for nonsmall cell lung cancer[30,31] and the predominant subtypes of pathologically lung adenocarcinoma[32] and lung squamous cell carcinoma.[33]

Methylation markers have already been used in clinical practice. Septin 9 (SEPT9) methylation testing is the first FDA-approved epigenetic biomarker for colorectal cancer screening.[34,35] Table 1 summarizes clinically available methylation-based detection methods that aided in clinical decision-making. Additionally, numerous methylation markers currently in development have demonstrated promising diagnostic performance. Two methylation markers, TMEFF2 and NGFR, were consistently detected in CRC patients.[35] DNA methylation alterations in GSTP1 and RASSF1 genes have emerged as potential biomarkers for the early diagnosis of prostate cancer and hepatocellular carcinoma (HCC), respectively.[36,37] Early lung squamous cell carcinoma (LUSC) detection was achieved by analyzing methylation abnormalities in TBX4, TRIM15, C6orf201, ARHGEF4, and OR4D11.[33] The methylation of RASSF1A, GSTP1, RARB, and the promoter of the SRY-box 17 (SOX17) gene was used to detect early stage breast cancer.[38,39]

2.2. Histone Modifications

Beyond DNA methylation, abnormal post-translational histone modifications (PTMs) and altered expression of histone variants (including H2A.Z, macroH2A, H2A.B, and H3.3) have emerged as significant biomarkers in cancer diagnostics. Histones play a vital role in eukaryotic chromosomes, with their exposed N-terminals mediating interactions with regulatory factors and serving as hotspots for post-translational modifications. Histone modifications encompass diverse processes, including methylation, acetylation, phosphorylation, ubiquitination, and SUMOylation. These site-specific histone modifications serve as conduits for signaling pathways, ultimately inducing transcriptional changes such as transcrip-

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tional activation or repression.[79] Histone modifications regulate biological events by various proteins and enzymes, such as writers, erasers, and readers, which can impact the development of cancer (Figure 2c).[17] In normal cells, DNA regions containing tumor-suppressor gene promoters are marked by specific histone modifications, such as acetylation on multiple lysine residues of histones H3 and H4 (notably at positions K5, K8, K9, K12, and K16) and H3K4me3.[80] In cancer, disruption of the normal pattern of histone modifications leads to the inappropriate activation of oncogenes.[80] This occurs by losing "active" histone marks on tumor suppressor genes and removing repressive marks, such as H4K20me3 and H3K27me3, from DNA regions that should remain silenced, including specific repeats at chromosome ends.

Abnormal histone modifications misregulate gene expression, impairing essential processes like transcription, cell division, programmed death, and DNA repair, which can culminate in oncogenic transformation. The analysis of histone PTMs and their variations in the context of the tumor environment can be used as biomarkers for diagnosis. For example, genetic alteration resulting in p300 HAT gene loss of heterozygosity has been related to hypoacetylation in different cancer cells and primary tumors.[81,82] Loss of H4K16ac and H4K20me3 is reported as an early alteration of nonmelanoma skin cancer.[82] H3K4me, H3K9me2, H3K9me3, H3Ac, and H4Ac were markedly decreased in malignant prostate tissue compared to normal prostate tissue.[83] Biomarkers of nonsmall cell lung carcinoma reveal an abnormal pattern in cancer cells, characterized by hyperacetylation of histones H4K5 and H4K8 and hypoacetylation of histones H4K12 and H4K16 compared to normal lung epithelium.[84] H4K20me3 is reported as a potential biomarker for early detection, and H4K20me3 loss occurred more commonly in squamous cell carcinoma than in adenocarcinoma.[84]

Epigenetic downregulation of H3K4me2 and H3K18ac correlates with poorer survival probabilities of lung, kidney, and prostate cancer.[85] In addition, H3K9me2 is associated with both gene activity and repression, and its reduced cellular levels serve as a prognostic indicator of poorer outcomes in patients with prostate or kidney cancers.[85] Lower histone H2AK5ac expression in pathologic stage II lung carcinoma is significantly associated with worse patient survival outcomes.[86] High H3K4me2 expression is associated with improved survival in patients with Stage I large cell or squamous cell lung cancer. Histone modification alterations as cancer biomarkers are still under clinical development. A promising clinical application involves reducing H3K27me3 expression as a supplementary diagnostic tool to differentiate melanoma from atypical proliferative nodules in children.[87]

2.3. Chromatin Accessibility

Chromatin accessibility reflects the functional availability of genomic DNA for molecular interactions, governed by the compaction state of chromatin, particularly TFs, regulatory proteins, and the transcriptional machinery. Eukaryotic genomes are generally packed into nucleosomes, a core chromatin element containing ∼ 147 bp of DNA tightly associated with a histone octamer. Regulated nucleosome positioning creates differential chromatin accessibility patterns that are cell type-specific. The composition and post-translational modification of nucleosomes are frequently linked to chromatin accessibility, with their dynamics providing insights into functional states through various mechanisms, including the characteristic distribution of open and closed chromatin regions, which arises from the combined effects of multiple cellular activities like chromatin remodeler activation, transcription, DNA damage repair, and replication (Figure 2d).[88] Permissive chromatin, unlike its closed counterpart, is highly flexible, permitting transcription factors to remodel accessibility in a sequence-dependent manner and establish an open chromatin conformation. Although nucleosomes are densely organized in facultative and constitutive heterochromatin, their distribution across the genome is highly dynamic, with significant reductions at key regulatory regions, including enhancers, insulators, and transcribed genes.

Alterations in the chromatin landscape, along with mutations in chromatin remodelers and regulatory patterns, are associated with cancer.[89] Chromatin accessibility is cell type-dependent; the measurement of chromatin accessibility can reflect cancer-associated alterations in cellular makeup, gene regulation, and epigenetic states. These modifications regulate changes in chromatin accessibility, serving as a key mechanism through which histone acetylation influences tumor progression, either positively or negatively. Histone acetyltransferase (HAT) (P300/CBP, MYST family, and GNAT family) influences particular lysine sites on histones, which neutralize the electropositive character of lysine residues, attenuating the electrostatic attraction interaction between histones DNA/neighboring histones, making chromatin more accessible.[90] Histone acetylation compromises charge-mediated histone-DNA contacts, which results in an open chromatin conformation, thus facilitating the accessibility by biomolecules like TFs or protein modules.

Alterations in histone methylation patterns, such as H3K4me and H3K79me, appear to be associated with tumor suppression. Increased levels of H3K9me modification enhance chromatin accessibility, aiding in the prevention of hepatocellular carcinoma progression.[91] Alpha/Beta-Hydrolase Domain Containing 5 (ABHD5) is recognized as an essential regulator of tumor suppressor gene in CRC, ABHD5 loss enhances chromatin openness thereby facilitating YAP-induced transcription of c-Met synergistically, consequently enhancing the stemness of CRC.[92] Some research has revealed that genes encoding ubiquitinate govern tumor progression by chromatin accessibility modulation. With BRCA1-Associated Protein 1 (BAP1) mutation, chromatin accessibility displayed preferential localization at TSS regions, with accessible regions clustered in functionally relevant genomes containing essential cell junction components.

3. SEQUENCING TECHNOLOGIES FOR CANCER EPIGENOMIC ANALYSIS

3.1. DNA Methylation Sequencing

3.1.1. Bulk DNA Methylation Sequencing. Bisulfite sequencing (BS-seq) is the benchmark technique for detecting DNA methylation in bulk samples. This method relies on bisulfite treatment, which turns unmethylated cytosines into uracils, whereas methylated cytosines remain unaffected.[93] Two major technologies have been developed based on bisulfite chemistry: whole-genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS).[94] These two technologies differ mainly in coverage and cost. WGBS provides comprehensive genome-wide coverage, but it requires significantly more sequencing reads

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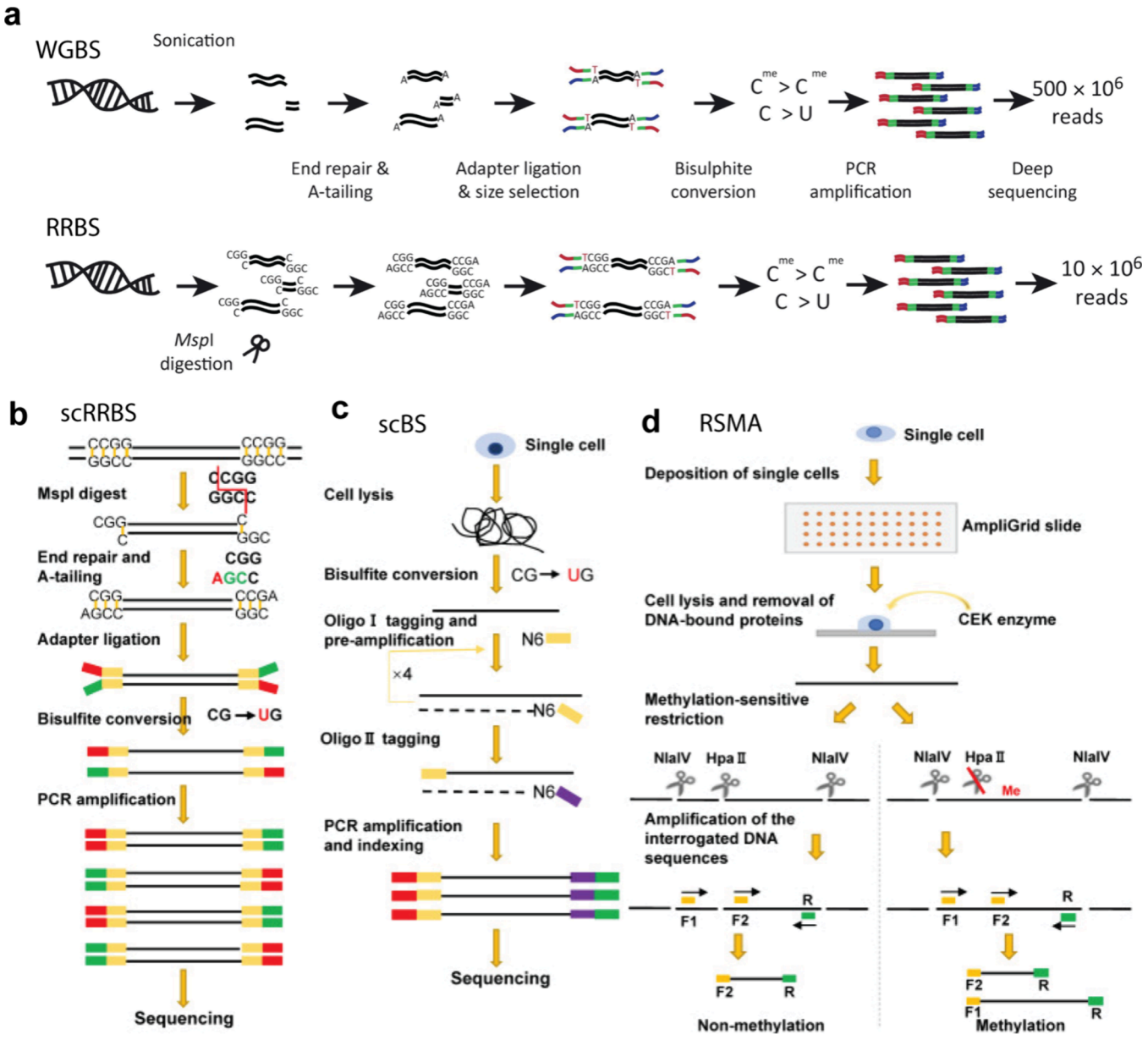

Figure 3. (a) Whole-genome bisulfite sequencing (Top) and reduced representation bisulfite sequencing (Bottom). Reproduced from ref 95. Copyright 2018 Cell. Illustrated of single-cell DNA methylation detection approaches: (b) scRRBS, (c) scBS-seq, (d) and RSMA. Reproduced from ref 100. Copyright 2023 NCBI.

to achieve adequate coverage, increasing costs and computational resources. While RRBS focuses on a subset of the genome, typically targeting CpG-rich regions, but missing large portions of the genome, particularly CpG-poor regions. Both these methods rely on bisulfite conversion of the DNA to distinguish unmethylated cytosines from methylated ones, they vary marginally in how the samples are processed for sequencing analysis. Sonication is generally applied to shear the DNA into random fragments in WGBS (Figure 3a).[95] These fragments are then subjected to end repair, undergoing end blunting followed by 3′-end adenylation, a process known as A-tailing. The overhanging adenosines are binding sites for sequencing adapters ligated onto the DNA fragments. The appropriately sized DNA fragments are size-selected and treated with bisulfite, inducing deamination of unmethylated cytosines to uracil, while preserving methylated cytosines. Bisulfite-treated fragments are PCR-amplified and sequenced, a minimum of 500 million reads are needed to achieve whole-genome coverage. In RRBS, enzymatic digestion generates DNA fragments with CG-rich terminal sequences. As a result, RRBS enables efficient profiling of CG-dense regions efficiently at economical sequencing requirements, which is more cost-effective than WGBS because it sequences only a fraction of the genome. These technologies allow precise identification of methylation at individual CpG sites. However, high sequencing costs and DNA degradation lead to reduced sequence quality, ultimately lowering the final reads yield. Postbisulfite adaptor tagging (PBAT) was developed to address these limitations.[96] Analyzing DNA methylation at CpG sites using PBAT in WGBS or measuring high CpG-content regions through RRBS is a widely used approach. While bisulfite treatment-based technologies are a powerful tool for DNA methylation analysis, the limitations of damaging DNA result in fragmentation, DNA loss, and biased sequencing data. Another common

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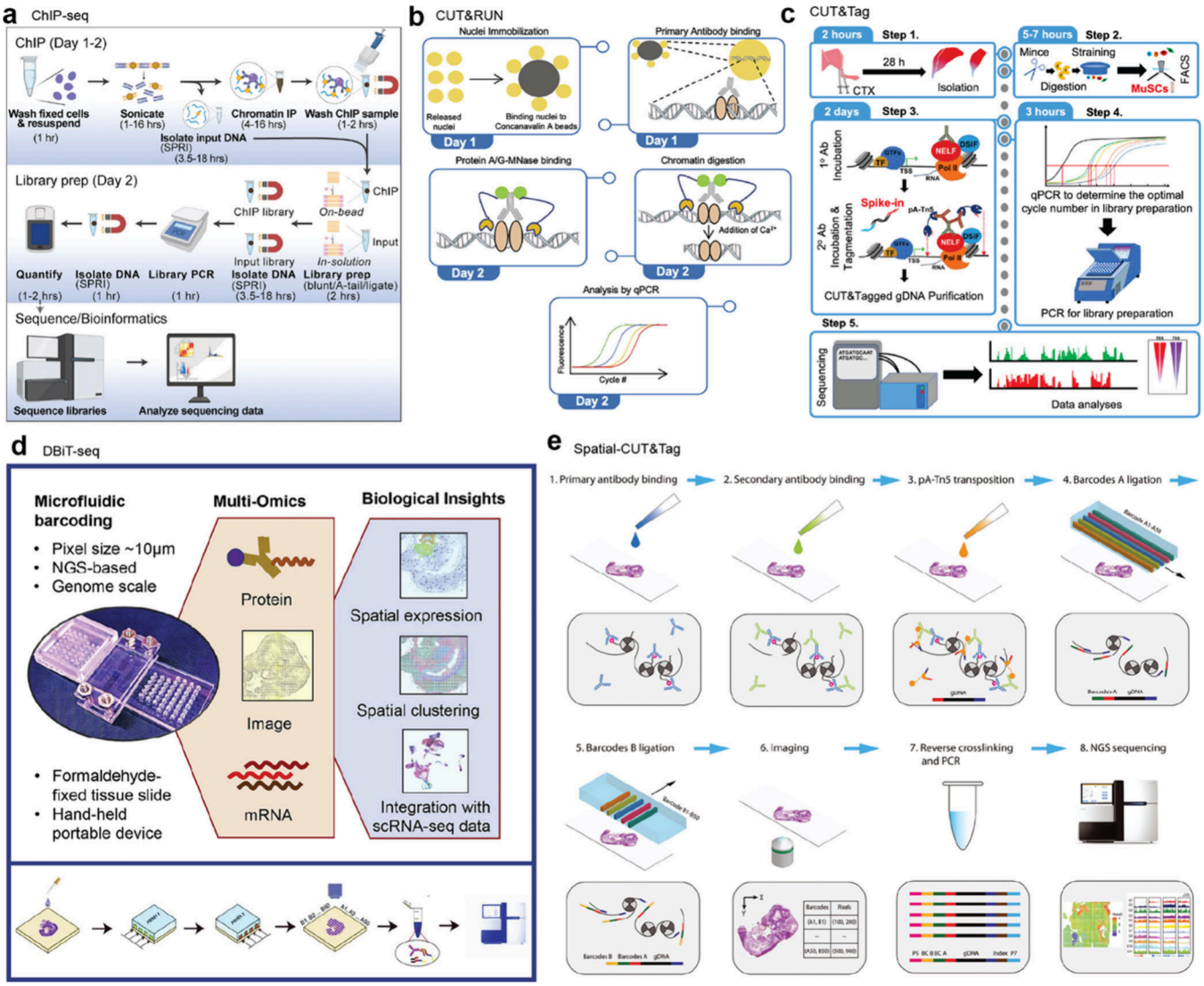

Figure 4. Procedure of chromatin profiling strategies. (a) ChIP-seq protocol. Reproduced from ref 110. Copyright 2021 Elsevier. (b) CUT&RUN protocol. Reproduced from ref 111. Copyright 2022 Elsevier. (c) CUT&Tag protocol. Reproduced from ref 113. Copyright 2021 Elsevier. (d) The device and workflow of DBiT-seq. Reproduced from ref 122. Copyright 2020 Cell. (e) The workflow of spatial-CUT&Tag sequencing. Reproduced from ref 121. Copyright 2022 Science.

drawback is the difficulty in discriminating between 5mC and its oxidative derivatives, such as 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC). This is because bisulfite processing converts unmethylated cytosine and 5fC and 5caC into uracil, which is then read as thymine during sequencing. Bulk methodologies helped revolutionize our knowledge about DNA methylation. However, bulk sequencing has high cell input needs (1×10^3-1×10^6 cells) and the population-averaging effect prevents tumor epigenetic heterogeneity analysis.

3.1.2. Single-Cell DNA Methylation Sequencing. To meet the need for high-resolution measurements on small-scale samples, several technologies have been developed based on bulk methodologies for single-cell DNA methylation analysis. Numerous dimensions of cancer heterogeneity that were previously inaccessible to analyze are now unlocked for reach. The first single-cell DNA methylation methodology was proposed in 2013, single-cell reduced representation bisulfite sequencing (scRRBS),[97,98] which utilizes restriction enzyme treatment to excise DNA fragments with CpG-rich ends, adapting the single-tube RRBS strategy (Figure 3b).[95] The method demonstrates exceptional sensitivity and is capable of profiling the methylation status across 1.5 million CpG loci in single stem cells. Since then, Numerous single-cell technologies utilizing bisulfite conversion have been established for DNA methylation sequencing. scBS-seq is an accurate and reproducible technique to characterize the methylome of rare cell types and heterogeneous populations.[99] In scBS-seq, sequencing adaptors are ligated after bisulfite treatment, yielding DNA fragmentation and unmethylated cytosines to thymine (Figure 3c).[100] Despite being complementary, scBS-seq achieves ~ 5-fold more CpGs and ~ 1.5-fold more CGI coverage versus scRRBS at equivalent sequencing depth. WGBS allows DNA methylation mapping in tiny cell populations (μWGBS) and single cells (scWGBS),[9] the entire postbisulfite library preparation is performed in one tube, reducing DNA loss and contamination risk. Compared to the scRRBS protocol, which focuses on CpG islands, scWGBS offers a more unbiased approach and achieves cumulative coverage of over 90% genomic CpG sites. To enhance the

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efficiency of single-cell capture, a digital-scRRBS version was developed.[101] Digital-scRRBS is the first microfluidic single-cell methylome library platform. Digital-scRRBS uses a microfluidic chip for single-cell isolation within 15 s, achieving single-cell methylome libraries with 53.6% unique mapping and up to 2.26 million CpG sites. Meanwhile, to reduce sequencing expenses and avoid potential amplification biases, a new technology named scPBAT was developed, which is the first repeat-optimized, PCR-free single-cell methylome sequencing approach.[102]

In addition to bisulfite-based approaches, multiple methylation-sensitive restriction enzymes (MSREs)-based, nonbisulfite methods have emerged. The restriction enzyme-based single-cell methylation assay (RSMA) was the first to profile CpG methylation at specific loci (Figure 3d).[103] Thereafter, single-cell restriction analysis of methylation (SCRAM) combines MSRE digestion and multiplex PCR for single-cell methylation analysis.[104] This method integrates a microfluidic qPCR platform enabling simultaneous DNA methylation assessment at 24 genomic loci across 48 cells per run. Single-cell methylase-assisted bisulfite sequencing (scMAB-seq) is built upon traditional bisulfite sequencing methods, which first treat with a methyltransferase to label unmodified cytosines, ensuring only endogenous methylation is preserved. Then, bisulfite conversion and sequencing distinguish the original 5mC (retained as C) from unmethylated sites (converted to T), enabling genome-wide and accurate profiling of methylation patterns.[105] Subsequently, enzyme conversion-based treatment methods, such as enzymatic methyl-seq (EM-seq)[106] and sciEM,[107] have been developed as the less destructive alternative to single-cell analysis. Recent advances enable high-throughput single-cell methylome profiling via combinatorial indexing. Single-cell combinatorial indexing for methylation analysis (sci-MET) utilizes a streamlined workflow involving fluorescence-activated nuclei isolation, followed by Tn5 tagmentation and PCR amplification, ultimately enabling NGS.[108] Similarly, single cell-targeted analysis of the methylome (scTAM-seq), a commercial microfluidic platform for targeted, bisulfite-free methylome analysis, can directly cover 650 selected CpG sites in up to 10,000 cells.[109] Numerous techniques profile DNA methylation at a single-cell resolution. However, several challenges must be addressed before it can be effectively used for cancer diagnostics, including low throughput, limited coverage per cell, and high costs.

3.2. Profiling of Histone Modifications

3.2.1. Bulk Histone Modifications. Histone post-translational modifications regulate chromatin states for transcriptional activation or repressive. Chromatin immunoprecipitation sequencing (ChIP-seq) has emerged as the most popular method for histone modification mapping. Using antibodies against specific DNA-binding proteins or histone marks, it identifies genome-wide enriched loci (Figure 4a).[110] While analyzing fewer than 10,000 cells is technically feasible, constructing a library from limited genomic DNA proves challenging due to incomplete recovery of bound DNA. Cleavage under targets and release using nuclease sequencing (CUT&RUN-seq) is another approach performed on intact, unfixed permeabilized cells or nuclei. The process involves incubating the cells with specific antibodies, followed by protein A-Micrococcal Nuclease (pA/MNase) fusion protein binding (Figure 4b).[111] Unlike ChIP-seq, which requires cross-linking and demands a large number of samples, CUT&RUN is simpler and more robust than CHIP, producing minimal background signals while requiring only 10% of the sequencing.[112] A recent development of chromatin profiling technology, CUT&Tag, was further developed to reduce the sample input. In the CUT&Tag technique, the Protein A-Tn5 transposase is used instead of micrococcal nuclease (MNase) as in CUT&RUN (Figure 4c),[113] essentially allowing for simultaneous cleavage of chromatin at antibody-bound sites and the addition of sequencing adapters during the "tagmentation" step, which can efficiently profile chromatin features in tiny samples.[114]

3.2.2. Single-Cell Histone Modifications. The single-cell resolution of these histone marks can help uncover regulatory heterogeneity among cancer cells, allowing for rare chromatin state analysis that may be linked to cancer diagnostics. Two technologies for analyzing single-cell histone modifications are Drop-ChIP/scChIP-seq[115] and scDamID.[116] Single-cell ChIP-seq (scChIP-seq) is a histone marks analysis method that can help properly profile and dissect regulatory heterogeneity at the single-cell level.[115] scChIP-seq enables single-cell chromatin profiling via microfluidics and DNA barcoding. Cells are lysed and micrococcal nuclease (MNase)-treated in drops, then barcoded and immunoprecipitation. This process enhances pull-down efficiency, leading to reduced background noise. A ChIP-based single-cell modification technique, known as simultaneous indexing and tagmentation-based ChIP-seq (sc-itChIP-seq), a cost-effective and efficient method, combines chromatin accessibility, cellular barcoding, and tagmentation in a single tube.[117] scChIP-seq performs cellular barcoding through MNase digestion and ligation in microfluidics droplets. In contrast, sc-itChIP-seq utilizes Tn5 transposase tagmentation coupled with chromatin opening to introduce cellular barcodes. These methods enable the analysis of 10−1000 single cells per run, generating 1,000−10,000 distinct reads per cell.

Single-cell chromatin immune-cleavage sequencing technique (scChIC-seq) is adapted from the CUT&RUN-seq In scChIC-seq, adapter ligation is performed on all retrieved DNA fragments to minimize DNA loss, while subsequent PCR amplification is designed to be specific for target fragments only.[118] Other techniques involving sequencing of epigenomic modifications are scCUT&Tag (single-cell CUT&Tag),[114] single-cell cleavage under targets and release using nuclease (scCUT&RUN),[119] combinatorial barcoding and targeted chromatin release (COBATCH), antibody-guided chromatin tagmentation sequencing (ACT-seq),[120] single-cell chromatin integration labeling sequencing (scChIL-seq). ACT-seq uses a Tn5 Protein A fusion to fragment chromatin and insert tags at antigen-specific genomic sites. The Tn5 transposase powers an index multiplexing strategy (iACT-seq), which can efficiently construct thousands of single-cell libraries independent of droplet microfluidics or FACS sorting. Plate-based split-pool barcoding elevates single-cell histone marks profiling from hundreds to tens of thousands of cells per run.[120] The Tn5-based approach streamlines high-throughput single-cell epigenomics analysis by concurrently integrating tagged sequences. The enzyme-tethering single-cell methods make it possible to detect thousands of unique reads per cell.

3.2.3. Spatial Histone Modifications. Despite the breakthroughs in single-cell histone modification detection, capturing the spatial information on cells in complex tumor environments remains challenging. Rather than placing the

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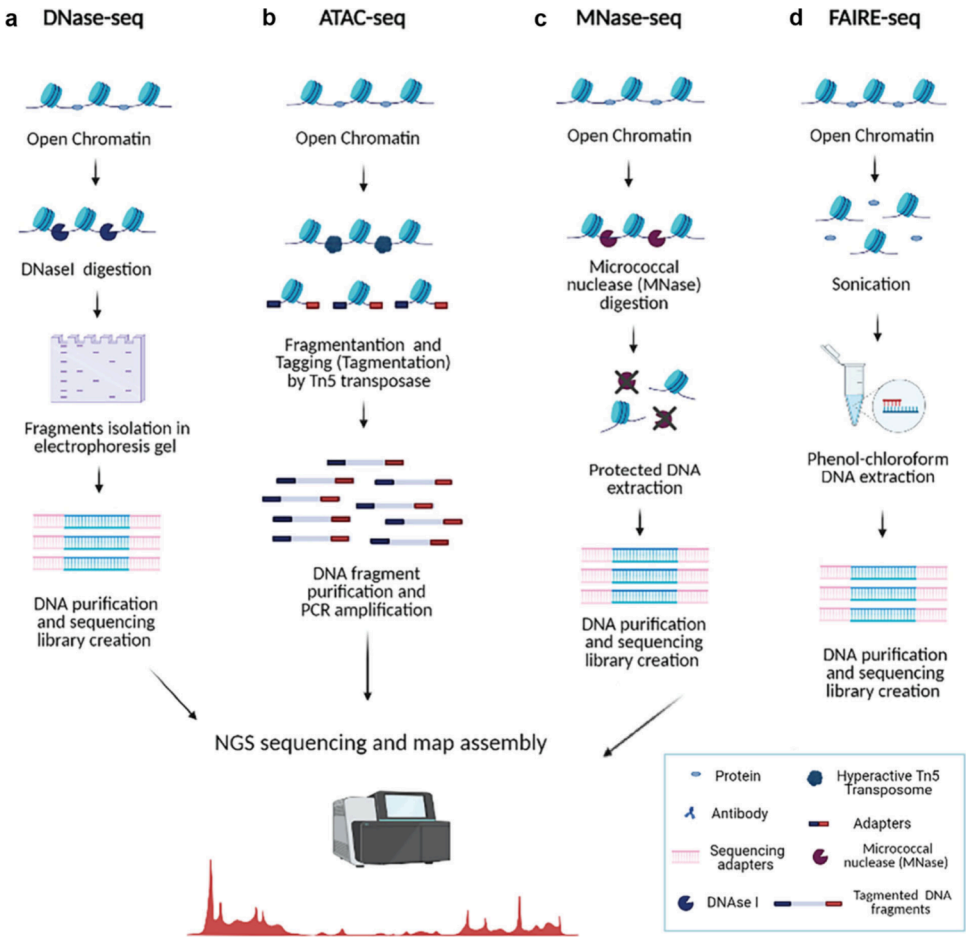

Figure 5. Principal techniques for mapping chromatin accessibility. (a) In the DNase-seq workflow, DNase I cleaves accessible chromatin, generating fragments. (b) In ATAC-seq, Tn5 transposase inserts adapters (the red and blue lines) to accessible sites, resulting in fragments directly amplified by sequencing libraries. (c) MNase-seq utilizes MNase to digest protein-free DNA regions while preserving protein-bound DNA fragments. (d) FAIRE-seq works by extracting fragmented DNA un-cross-linked to nucleosomes. Reproduced from refs 124 and 129. Copyright 2021 MDPI.

spatial barcodes onto chips, spatial-CUT&Tag was recently developed for whole-genome profiling of histone modifications.[121] A primary antibody targeting histone mark is treated in a fixed tissue section and then detected with a secondary antibody (Figure 4d, e).[121,122] Following pA-Tn5 transposome activation, genomic DNA was tagged with adapters containing a ligation linker at histone mark antibody recognition sites. Utilizing a custom microfluidic barcode delivering method, deterministic barcoding in tissue for spatial omics sequencing (DBiT-seq),[122] spatial-CUT&Tag reveals many key regulatory elements such as H3K27me3 (repressing loci), H3K4me3 (activating promoters), and H3K27ac (activating enhancers and/or promoters).[121] Comparative analysis reveals spatial-CUT&Tag at the 20-μm resolution achieves significantly higher unique fragments (H3K27me3:9735; H3K4me3:3686) versus scCUT&Tag (H3K27me3:682; H3K4me3:453) on mouse brain at equivalent sequencing depth. This technology enables high-resolution spatial profiling of histone marks and brings epigenetics research into the spatial era. Spatial mapping of chromatin modification in tissue opens new avenues for investigating epigenetic regulation, cell behavior, and differentiation in health and disease.

3.3. Chromatin Accessibility Sequencing

3.3.1. Bulk Chromatin Accessibility Sequencing. Whole-genome DNA accessibility assays facilitate the detection of DNA regulatory regions including promoters and enhancers, providing insights into epigenomic alterations that modulate gene expression activation or silencing. In 2008, the first method to profile genome-wide accessible chromatin was published, named DNase I hypersensitive site sequencing (DNase-seq).[123] DNase-seq combined high-throughput sequencing with genome-wide tiling arrays to detect DNase I hypersensitive (HS) sites generating a precise whole-genome chromatin accessibility map (Figure 5a).[124] DNase I selectively cleaves accessible chromatin, producing fragments that are subsequently amplified into sequencing libraries. DNase-seq has several limitations, including the need for a large input of cells (tens of millions) and a lengthy, labor-intensive protocol that takes several days to complete.[125] Thereafter, many more methods were developed. ATAC-seq has been established as an efficient method for studying accessible chromatin profiles by directly inserting sequencing adaptors into native chromatin. This approach has been developed into a rapid and sensitive technique for integrative epigenomic analysis (Figure 5b).[124,126] ATAC-seq uses a hyperactive Tn5 transposase to target and fragment accessible chromatin while

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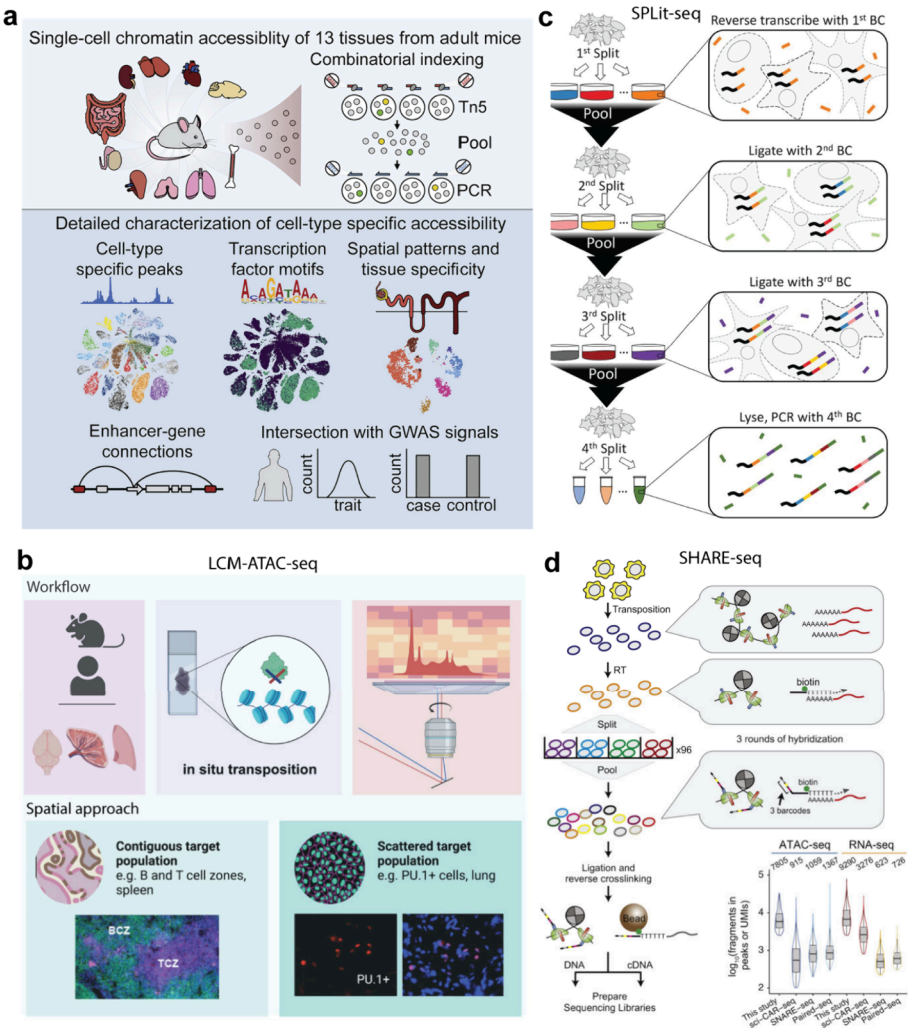

Figure 6. (a) sci-ATAC-seq workflow and functional annotation of major regulators and regulatory sequences in heterogeneous cell populations. Reproduced from ref 138. Copyright 2018 Cell. (b) Spatial approach of LCM-ATAC-seq. Reproduced from ref 141. Copyright 2023 Cell. (c) Overview of SPLiT-seq. Reproduced from ref 149. Copyright 2018 Science. (d) Schematic of the SHARE-seq protocol for integrated analysis of scATAC and scRNA within the same cell. Reproduced from ref 150. Copyright 2020 Cell.

adding sequencing adapters, enabling direct PCR amplified into libraries. DNase-seq and ATAC-seq both produce peaks in sequencing reads at accessible regions. The primary advantage of ATAC-seq and its newer variants lies in their high sensitivity, allowing effective analysis of low-input samples (Only 500−50,000 cells are needed versus millions for DNase-seq).

Additionally, they utilize an operationally efficient protocol by combining chromatin fragmentation and tagging in a single step. Beyond accessible chromatin mapping, ATAC-seq reveals TF footprints and nucleosome positions. MNase-seq is the most commonly employed technique for profile nucleosome occupancy genome-wide, which plays a critical role in chromatin accessibility.[127] In MNase-seq, micrococcal nuclease cleaves unprotected DNA but leaves intact protein-bound fragments (especially nucleosomal DNA) (Figure 5c).[124] MNase chromatin typically produces a nucleosome ladder consisting of mononucleosome, dinucleosome, trinucleosome, etc., varying with MNase-to-cell ratio. MNase-seq library preparation requires 10,000−100,000 fresh or fixed cells. Like DNase-seq, MNase-seq requires precise enzyme titration and involves a two-day protocol. FAIRE-seq (Formaldehyde-Assisted Isolation of Regulatory Elements) provides an alternative approach for identifying regulatory genomic regions and has been successfully applied to various eukaryotic cells and tissue types. Cells or dissociated tissues are first cross-linked with formaldehyde, then lysed and sonicated (Figure 5d).[124] Sheared chromatin undergoes phenol/chloroform extraction, yielding purified DNA, typically encompassing 1−3% of the human genome for library creation.[128]

3.3.2. Single-Cell Chromatin Accessibility Sequencing. Chromatin accessibility varies by cell type, and significant heterogeneity is often present in tumor samples. To avoid masking this variability through population averaging, it is recommended to analyze accessible chromatin landscapes at the single-cell resolution. Recently, a single-cell DNase-seq assay (scDNase-seq) based on DNase-seq has been proposed to analyze single or small cells.[130,131] This technology requires merely hundreds to thousands of either fresh or fixed cells, completes library construction in 24 h, and bypasses DNA fractionation.[132] single-cell ATAC-seq (scATAC-seq), the current gold standard for single-cell DNA accessibility analysis, utilizes either droplet microfluidic or fluorescence cytometry/plate-based sorting for nuclei isolation. The two popular

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commercially available solutions for microfluidic-based scATAC are the Chromium Next Gem system from 10x Genomics[133] and the SureCell platform from BioRad.[134] However, these commercial technologies require sample processing devices that are not routinely available in most laboratories. Plate-based scATAC-seq provides a nonmicrofluidic alternative, where individual cells are physically separated into plate wells. Standardized 96 and 384-well protocols,[135] provide experimental simplicity but are inherently limited in throughput capacity. By adapting scATAC-seq to the ICELL8 platform (Takara Bio) with 5,084 nanolitre wells, a method termed μATAC-seq, achieved throughput to several thousand cells per run.[136] Single-cell combinatorial indexing ATAC-seq (sciATAC-seq) demonstrates that throughput limitations can be overcome through innovative barcoding strategies.[137]

The combinatorial cellular indexing-based methods like sciATAC-seq can be used to profile chromatin accessibility, identifying 85 distinct patterns across ∼ 100,000 single cells from 13 adult mice, with most cell types, successfully classified into specific types and ∼ 400,000 differentially accessible elements detected (Figure 6a).[138] This two-step barcoding workflow first labels nuclei during in-plate tagmentation, and then introduces a second barcode after plate transfer. The dual-indexing system enables precise single-cell identification through combinatorial barcode matching. ATAC-seq methods detect chromatin accessibility by leveraging Tn5 transposition to simultaneously fragment and tag open genomic regions with sequencing adapters.[139] Additionally, single-cell micrococcal nuclease sequencing (scMNase-seq) provides insights into chromatin compaction and nucleosome positioning at single-cell resolution. This is achieved by using MNase to digest the linker DNA between nucleosome cores, followed by sequencing the protected DNA regions.

3.3.3. Spatial Chromatin Accessibility Sequencing. To preserve the in situ spatial organization of target cells or nuclei within tissue structures and link epigenetics with spatial information, sciMAP-ATAC (single-cell combinatorial indexing on microbiopsies for transposase-accessible chromatin) utilizes microbiopsy punching combined with combinatorial indexing for spatially resolved scATAC-seq.[140] While sciMAP-ATAC maintains single-cell chromatin accessibility profiling quality equivalent to nonpatterns sci-ATAC-seq and preserves cellular spatial information in intact tissues, its resolution is constrained by 214-μm cubic microbiopsies. Additionally, tissue morphology analysis requires posthoc alignment with separately stained sections. Various methods involve using tailored tools to precisely identify and extract regions of interest from samples for downstream analysis. Laser capture microdissection coupled with ATAC-seq (LCM-ATAC-seq) is an advanced technique for spatially characterizing chromatin accessibility. This method employs ultraviolet (UV)-based laser capture microdissection (LCM) to selectively extract targeted cell populations from tissue sections, combining microscopic visualization with precise laser dissection. It enables the collection of samples with spatial single-cell-level information for profiling chromatin accessibility (Figure 6b).[141] This spatial methodology allows for mini-bulk level analysis of dispersed cell populations in tissues while retaining compatibility with cellular or morphological staining on the same specimen.

A novel approach named spatial-ATAC-seq has recently been developed, offering a fundamentally different method for spatially resolving chromatin accessibility. Instead of isolating cells with spatial information, this technique combines in situ Tn5 transposition chemistry with flow barcoding to profile chromatin accessibility directly within intact tissue sections.[142] With spatial-ATAC-seq, accessible genomic regions and their regulatory factors within defined tissue architectures can be simultaneously localized. Subsequently, a method called solid-phase-capture spatial ATAC-seq was introduced, integrating chromatin accessibility mapping with tissue sections with barcoded solid-phase capture. This approach enables spatial epigenomic analysis, serving as an epigenomic counterpart to the spatial transcriptomics (ST) method.[143] First, fresh frozen tissue sections are affixed to barcoded slides and cross-linked to maintain chromatin integrity throughout immunostaining procedures. Following immunostaining, tissue sections are imaged to acquire tissue coordinates and protein expression profiles. Subsequently, Tn5 transposase is applied to permeabilized sections for open chromatin. During gentle tissue digestion, DNA fragments are hybridized to spatially barcoded surface oligonucleotides using a chimeric splint oligonucleotide. Splint ligation and polymerase extension tag open chromatin with spatial barcodes. PCR handles are then added to create a sequencing library. This solid-phase-capture spatial ATAC-seq method, based on in situ transposition and spatial barcoding, can resolve spots with a 55 μm diameter.

3.4. Integrating Single-Cell Epigenomics with Other Omics Technology

Complex cancer systems are challenging to fully understand using bulk analyses or by examining individual layers of information in isolation. Therefore, integrating multiple single-cell omics technologies is essential to unveil the intricate mechanisms and gene regulation heterogeneity driving cancer development and progression. The breakthrough single-cell multiomics technology to simultaneously map the epigenome and transcriptome is single-cell methylome and transcriptome sequencing (scM&T-seq).[144] scM&T-seq splits single-cell RNA and DNA for separate analysis, using scBS-seq to detect DNA methylation. This parallel profiling allows comprehensive analysis of DNA methylation-transcription interplay across diverse cell populations. ScNOMe-seq is a powerful single-cell multiomics technology designed to simultaneously profile chromatin accessibility and DNA methylation within individual cells.[145] scNOMe-seq offers the advantage of sequencing reads being independent of accessibility measurements. This makes it particularly well-suited for characterizing chromatin organization in single cells within heterogeneous cellular samples. Another multiomics technology is single-cell triple omics sequencing (scTrio-seq), which simultaneously analyzes the genome, DNA methylome, and transcriptome through scRRBS and WGS.[146] Single-nucleus chromatin accessibility and mRNA expression sequencing (SNARE-seq) employ droplet microfluidics to simultaneously capture mRNA transcripts and chromatin accessibility from over 10,000 single cells in parallel.[147] An ultrahigh-throughput approach, Paired-tag, was introduced to coprofile histone modifications and transcriptome in individual cells, yielding detailed maps of chromatin state and transcriptome across distinct cell types within complex tissues.[148] Another Plate-based ultrahigh-throughput technology, SPLiT-seq (split-pool ligation-based transcriptome sequencing), involves randomly distributing fixed cells or nuclei into wells during each split-pool cycle, where transcripts are tagged with well-specific barcodes

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(Figure 6c).[149] A single SPLiT-seq experiment involving four pool-split rounds is capable of distinctly barcoding over 100,000 nuclei. SHARE-seq, an adaptation of SPLiT-seq and Paired-seq, enables high-throughput simultaneous profiling of ATAC and RNA expression within individual cellular units (Figure 6d).[150] SHARE-seq utilizes a 96-well plate format to label permeabilized cells or nuclei with unique barcodes that attach to both chromatin fragments and cDNA, generating approximately 10^6 (96^3) possible barcode combinations. Unlike single-cell methods for analyzing gene expression and chromatin accessibility, histone modification analysis methods suffer from limited sensitivity and throughput. ScCUT&Tag integrated with the scATAC-seq protocol, enables profiling histone modifications and chromatin occupancy of TFs at thousands of cells in complex tissue.[151]

3.5. Combining Spatial Epigenomics with Complementary Techniques

Single-cell methods for epigenomic profiling face limitations in achieving genome-wide coverage while retaining spatial resolution. Spatially resolved approaches are required to enable large-scale simultaneous gene expression and regulatory genomic information profiling within their native tissue context. The DBiT platform enables the expansion from spatial single omics to multiomics sequencing and offers an efficient approach for high-resolution profiling of complex cells and tissues. Spatial ATAC&RNA-seq have transformed whole-genome coprofiling of chromatin accessibility and mRNA expression, while spatial CUT&Tag-RNA-seq extends this capability by integrating histone modification mapping with transcriptomics sequencing. These two technologies combine spatial ATAC-seq or CUT&Tag with transcriptomics in a single tissue utilizing DBiT strategies, achieving simultaneous cellular-level profiling.[152] Microfluidic indexing-based spatial assay for transposase-accessible chromatin and RNA-sequencing (MISAR-seq), a spatial multiomics method motivated by the DBiT-seq design and SHARE-seq methodology, was reported for coprofiling chromatin accessibility and transcriptomes using microfluidic indexing.[153] To capture information from multiple molecular layers, two novel spatial triomic sequencing technologies, DBiT ARP-seq (spatial ATAC-RNA-Protein-seq) and DBiT CTRP-seq (spatial CUT&Tag-RNA-Protein-seq), were developed alongside multiplexed immunofluorescence imaging (CODEX) achieving unprecedented insight into the molecular architecture of biological systems.[154] These approaches are especially valuable for deciphering the dynamic relationship between epigenetic changes and gene expression in complex physiological and pathological processes. To enable the spatial mapping of multiple layers of omics information, spatial-Mux-seq was developed. By combining spatial mapping with multimodal spatial technology, this method simultaneously interrogates two histone modifications, open chromatin, the whole transcriptome, and protein panels across entire tissues, maintaining cellular resolution with spatial context.[155] Slide-tags, a bead-based method, utilizes droplet-based combinatorial snATAC-seq and snRNA-seq to profile tagged nuclei, establishing a versatile system for single-cell multiomic measurements of open chromatin, RNA, and T cell receptor (TCR) sequences in metastatic melanoma.[156]

4. EPIGENOMIC SEQUENCING IN CANCER DIAGNOSTICS

Sequencing technologies for biomarker discovery can target pathologically and clinically significant epigenomic differences between interest groups. For instance, these technologies can compare nontumor and tumor samples to identify diagnostic biomarkers, high-risk and low-risk patient samples to uncover prognostic biomarkers and predictive biomarkers. Epigenomic sequencing technologies have facilitated the genome-wide detection of tumor-specific mutations and cancer-associated epigenetic patterns with high throughput. Their strength lies in generating comprehensive data sets, such as cell-type atlases and epigenomic landscapes. Establishing cellular lineage trees helps to characterize the cellular makeup of malignant and normal samples, identify novel cell types, and discover biomarkers. Numerous NGS-based epigenomic assay technologies, such as enhanced linear-splinter amplification sequencing (ELSA-seq), are registered for DNA methylation-based assays with clinical applications in oncology.[8] Epigenomic sequencing technologies enhance cancer diagnostics by facilitating the development of molecular biomarkers, deciphering cellular heterogeneity and its underlying mechanisms, and creating comprehensive cell lineage and atlas data sets.

4.1. DNA Methylation Sequencing in Cancer Diagnostics

4.1.1. Bulk DNA Methylation Sequencing in Cancer Diagnostics. Specific genes may exhibit abnormal methylation patterns in cancer cells long before morphological changes occur.[157] In addition to tissue samples, methylation signatures are measurable in liquid biopsies like blood, urine, and saliva, enabling noninvasive 'liquid biopsy' for oncological detection and monitoring. Bulk DNA methylation analysis generates averaged molecular signatures across samples. Examining CpG methylation at specific loci or assessing global methylation patterns has proven to be the most effective approach for identifying epigenetic biomarkers. The methods mentioned earlier, such as WGBS employed in clinical trials targeting a panel of over 100,000 informative methylation patterns in blood-based circulating cell-free DNA (cfDNA), enable highly specific multicancer detection (>50 types) and tissue origin localization across all stages.[74] The MCTA-seq method has identified thousands of hypermethylated hotspots in plasma cfDNA, effectively distinguishing HCC patients from cancer-free individuals.[158] MCTA-seq demonstrates high sensitivity, detecting with a minimum of 7.5 pg of DNA and identifying numerous precision markers for small-volume HCC (≤3 cm in size). Additionally, shotgun-based whole-genome bisulfite sequencing enables sensitive profiling of hypomethylation in plasma offering a robust strategy for detecting various cancer types. Bisulfite DNA sequencing provided genome-wide assessment of hypomethylation in the blood-derived DNA from patients diagnosed with HCC, breast cancer, nasopharyngeal cancer, lung cancer, smooth muscle sarcoma, and neuroendocrine tumor.[159] The findings revealed that plasma hypomethylation can be used to monitor hepatocellular carcinoma patients after tumor resection and to detect residual disease. ELSA-seq has been utilized for whole DNA methylome profiling to detect early stage cancers.[160] ELSA-seq enhances the analysis of cfDNA by minimizing artifacts associated with methylation sequencing. When integrated with a machine-learning algorithm for methylation pattern analysis, ELSA-seq surpasses other noninvasive methods in detecting

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low-frequency ctDNA in blood, offering significant potential for advancing clinical applications.

A bulk genome-scale DNA methylation analysis can unravel tumor heterogeneity and distinguish subtle distinctions from recurrent neoplasms. RRBS-seq has been employed to map the DNA methylation landscape correlated with glioblastoma disease progression.[161] These findings present both a comprehensive atlas of epigenetic heterogeneity in glioblastoma and a valuable repository of DNA methylation patterns with corresponding annotation data derived from large FFPE sample sets collected during routine diagnostics. Additionally, the study integrated DNA methylation analysis with diverse clinical, histopathological, and radiological information. Overall, bulk DNA methylation sequencing proves to be a robust method applicable to varied biosample collections for both diagnostic and clinical purposes.

4.1.2. Single-Cell DNA Methylation Sequencing in Cancer Diagnostics. The application of single-cell DNA methylation sequencing for cancer detection remains at a nascent stage. However, it holds great potential for exploring cellular heterogeneity, uncovering rare cell types, and identifying biomolecular markers. Single-cell locus-specific bisulfite sequencing (SLBS) has been successfully utilized to specifically single-cell analysis of DNA methylation epimutations.[162] The epigenetic profile collected from millions of cells reveals intercellular epigenetic heterogeneity and the pathogenic history of epimutations. DNA methylation profiles generated by WGBS and scBS-seq revealed partial methylation domains (PMDs) associated with CRC in individual cells, uncovering tumor heterogeneity. Furthermore, these single-cell PMDs offer valuable insights as potential epigenetic biomarkers previously obscured in bulk sequencing data.[163] Liquid biopsy enables access to circulating tumor cells (CTCs), which are essential for detecting metastases. Breakthroughs in single-cell analysis have revitalized interest in CTC research, providing valuable insights into metastatic processes. Methylation variability across individual CTCs was detected by the scBS-seq.[164] DNA methylation profiles demonstrated potential tumor origin classification, which addresses the epigenetic regulatory mechanisms underlying cancer metastasis and facilitates the future clinical application of CTCs.

An additional application of single-cell DNA methylation is constructing cellular lineage trees. scRRBS has been utilized to trace cancer evolution by analyzing DNA methylation patterns in both healthy donors and chronic lymphocytic leukemia (CLL) patients preand post-treatment.[165,166] Single-cell analysis reveals impaired B cell differentiation in CLL patients and highlights greater intercellular heterogeneity of malignant B cells compared to healthy individuals. The integration of transcriptome data reconstructed the clonal lineage of CLL and provided crucial information about tumor evolution. Single-cell DNA methylation data can uncover new cell types, enabling the differentiation between healthy and diseased cell populations. Single-cell multiomics technologies, like scTrio-seq[146] and scNMT-seq[167] can simultaneously decipher the multilayer bioinformation on single cells. By combining the methylation and transcriptome data of single cells, scTrio-seq was applied to analyze 25 single tumor cells isolated from HCC patients. This approach revealed two phenotypically distinct cell clusters by integrating single-cell CNVs, DNA methylome, and transcriptome profile.[146] Single-cell DNA methylation profiling also is a powerful tool for constructing cell-type atlases in cancer. These DNA methylation atlases can uncover fundamental patterns of cell type-specific methylation and characterize numerous cellular identity-determining regulatory architectures, providing a valuable resource for tumor diagnosis, classification, and more. Several research groups have published DNA methylation atlases of healthy tissue samples at single-cell resolution.[168−170] While single-cell methylation atlases for cancer have not yet been released, resources like the Cancer Genome Atlas (TCGA) offer cancer-specific epigenomic data that are valuable for cancer diagnosis.

4.2. Histone Modifications Profiling in Cancer Diagnostics

4.2.1. Bulk Histone Modifications Profiling in Cancer Diagnostics. Circulating histone modifications in circulating biofluids (e.g., plasma/serum) hold promise as biomarkers for cancer diagnosis and management. Efforts are underway to develop minimally invasive methods for analyzing histone modifications. A comprehensive analysis of 1,268 circulating epigenomic markers in plasma from 433 cancer patients (15 tumor types) was conducted using ChIP-seq and ATAC-seq platforms.[171] The data served as a reliable indicator of transcriptional activity, enabling prediction of diagnostic markers, identification of drug targets, and revelation of epigenetic resistance pathways. Epigenomic enhancer profiling can delineate cancer signatures, with the gain of enhancer activity being a common feature of cancers. Early studies used ChIP-seq to analyze genome-wide differences in active enhancers, with H3K4me identified as a marker for functionally active enhancers in primary colon cancer.[172] Another study combined ChIP-seq with genomic and RNA sequencing to map extensive enhancer enhancers networks and uncovered critical TFs associated with CRC development.[173] Genome-wide has confirmed the involvement of superenhancers in regulating PHF19 and TBC1D16 expression during CRC tumorigenesis, while simultaneously identifying KLF3 as an oncogenic transcriptional regulator in CRC. Through multimodal integration of transcriptomic profiles with chromatin mapping of androgen receptor (AR) along with three histone modifications (H3K4me3, H3K27ac, H3K27me3), successfully categorized prostate cancer into three molecularly defined subtypes.[174] By multiparametric analysis three epigenetic layers (histone modifications via ChIP-seq, transcriptomes, and methylomes) of pancreatic ductal adenocarcinoma (PDAC) subtypes were defined, whose epigenomic landscapes reflect varying degrees of malignancy and predict patient survival.[175]

4.2.2. Single-Cell Histone Modifications Profiling in Cancer Diagnostics. Single-cell histone modification profiling enables accurately characterizing chromatin features in cancerous cells, facilitating the identification of diagnostically relevant rare chromatin configurations. Technologies such as scChIP-seq have been used to characterize chromatin landscapes across thousands of cells with a coverage of up to 10,000 loci per cell.[176] This approach uncovered cell identities and distinguishing chromatin features, including activating (H3K4me3) and silencing (H3K27me3) marks, which modulate transcription in diverse single-cell populations. Applied to breast cancer patient-derived xenograft (PDX) samples, this approach characterized the heterogeneity of chromatin landscapes across both stromal and tumor cell populations. Histone modifications vary across different cancer stages and cell types, and determining cell type can aid in tumor staging and differentiate lesions from normal tissue. ScCUT&Tag for H3K27me3 can differentiate various heterogeneous blood cell types and derive PcG-mediated

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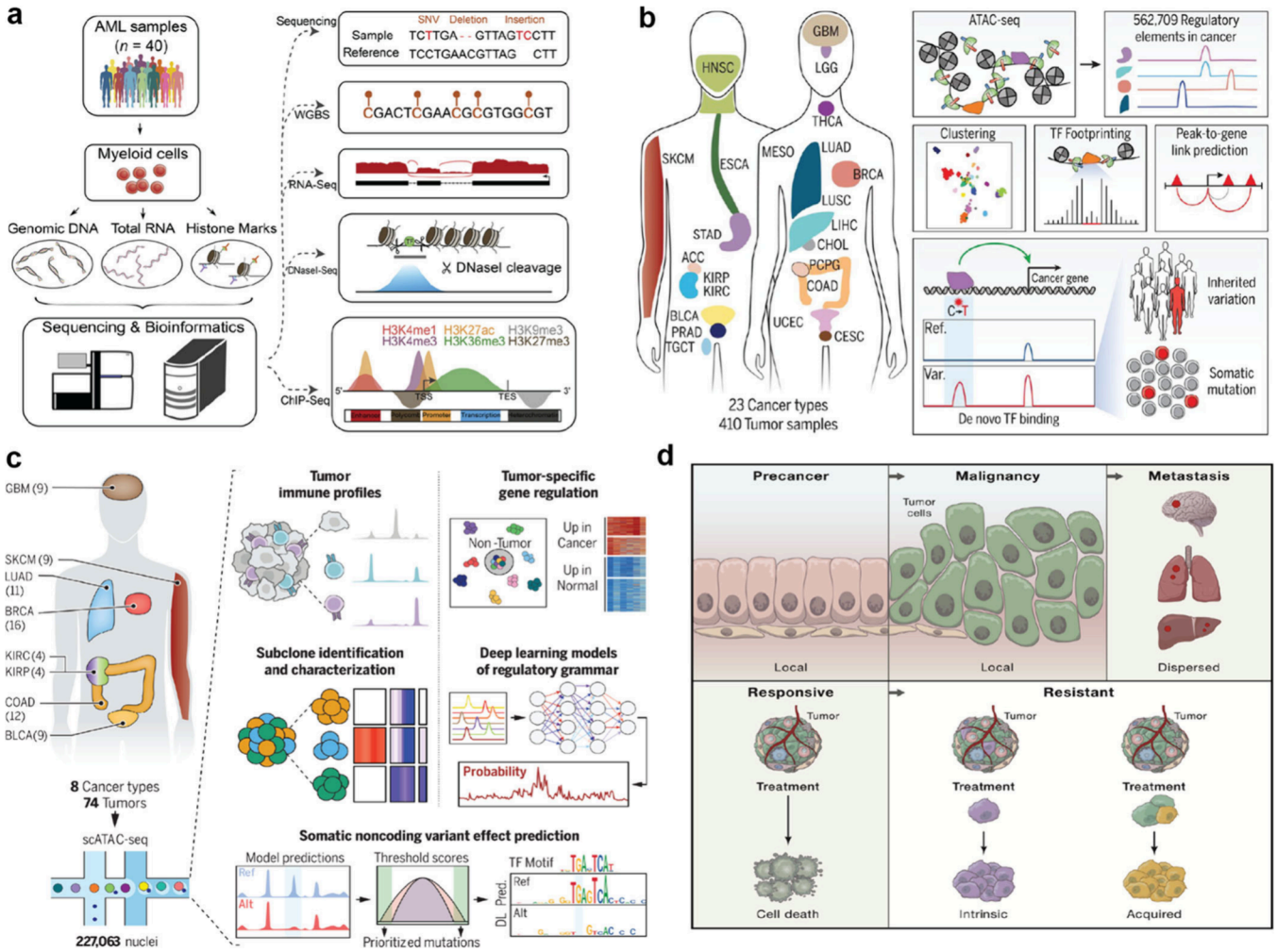

Figure 7. (a) Schematics of integrating ChIP-seq, RNA-seq, DNaseI-seq, and WGBS analysis of 38 representative AMLs samples capturing the abundant genetic heterogeneity enabled multiomics-based subtypes classification. Reproduced from ref 183. Copyright 2019 Cell. (b) Chromatin profiling of 23 cancers (410 TCGA tumors) revealed 562,709 DNA regulatory elements defining the cancer gene control landscape. Reproduced from ref 186. Copyright 2018 Science. (c) Single-cell chromatin accessibility atlas of pan-cancer gene regulation. Reproduced from ref 188. Copyright 2024 Science. (d) Core cancer transitions: initiation (across the premalignant-to-invasive carcinoma continuum), metastasis (from the localized-to-metastatic transition), and progression to resistance through intrinsic (purple) or acquired (yellow) mechanisms. Reproduced from ref 190. Copyright 2020 Cell.

epigenetic landscapes specific to each population.[177] Additionally, by performing scCUT&Tag to analyze H3K27me3 in a brain cancer patient pre- and post-treatment, different cell populations in the oncological ecosystem were identified and variations in PcG activity between the initial and post-therapy samples were uncovered. The lineage trees provide information about chromatin heterogeneity within patient samples, tumor-subtype-specific sites of aberrant chromatin regulation, and sensitivity to therapeutic agents. Technologies like automated scCUT&Tag can profile chromatin heterogeneity in mixed-lineage leukemia.[178] This study found that abnormal H3K4me3 accumulation in gene bodies responds to Menin inhibition, highlighting how automated chromatin profiling can reveal druggable targets. Integrating automated methods with scCUT&Tag enables the discovery of patient-specific epigenomic variation and the prediction of drug response patterns. These successful applications reinforce the value of the technologies mentioned above for studying histone modification patterns in individual tumor cells.

4.2.3. Spatial Histone Modifications Profiling in Cancer Diagnostics. To fully understand tissue organization, such as that of tumors and the brain, at a functional level, spatially resolved data provided by omics approaches is essential. Spatial distribution and genomic locations discovered are believed to regulate specific development-associated genes. Techniques like spatial-CUT&Tag, as discussed above, enable comprehensive profiling of histone marks across the genome, helping to identify the binding sites of specific proteins across the entire genome.[121] Spatial-CUT&Tag can reveal spatial patterns of histone marks such as H3K27me3 (which represses loci), H3K4me3 (which activates promoters), and H3K27ac (which activates enhancers and/or promoters). The spatial distribution of marker gene expression enables cell type discrimination. Unlike bulk or scCUT&Tag, the spatial variant provides information on the localization of proteins within tissues or cell types, offering insights into heterogeneous cellular populations and their unique regulatory landscapes. Although these technologies remain to be translated for direct tumor diagnosis, they hold great potential for exploring the

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spatial organization of chromatin in cancer tissues, revealing key features of the tumor microenvironment and mapping potential targetable pathways.

4.3. Chromatin Accessibility Sequencing in Cancer Diagnostics

4.3.1. Bulk Chromatin Accessibility Sequencing in Cancer Diagnostics. ATAC-seq offers valuable insights into leukemogenesis transformation, enabling the identification of early stage regulatory networks that represent potential therapeutic intervention points.[179] An ATAC-seq study demonstrated that Nuclear Factor I B (NFIB), a transcription factor, promotes metastasis by broadly increasing chromatin accessibility in human small-cell lung cancer.[180] Mutations in epigenetic modifiers early in development, followed by mutations in oncogenes that drive proliferation, serve as a critical regulator of the leukemogenic progression. By combining ATAC-seq with RNA-seq technology, widespread heterogeneity in the chromatin landscape of CLL was uncovered.[181] Chromatin accessibility mapping can reveal subtype-specific epigenome signatures and transcriptional regulatory networks in CLL. The integrating ChIP-seq, ATAC-seq, WGBS, and RNA-seq analyses delineated the reference epigenome and regulatory chromatin landscape of CLL, identifying widespread alterations in regulatory networks and providing insights into key connections between the genetic and epigenetic mechanisms in the disease.[182] Further characterization through integrating multilayer data sets, high-quality profiling using ChIP-seq, DNase-seq, RNA-seq, and WGBS reveals that acute myeloid leukemias with genetic heterogeneous can be stratified into two chromatin-defined categories, each exhibiting unique stemness characteristics (Figure 7a).[183] Beyond gene mutations, dysregulated enhancer activity in noncoding genomic regions plays a pivotal role in tumorigenesis. ChIP-seq and DNase-seq have revealed enhancers with repeatedly altered activity states in CRC specimens.[184] Recently, the ATAC-seq approach integrated with multiple-omics data sets constructed a comprehensive open chromatin accessibility architecture in nonsmall cell lung cancer and primary human cancers (Figure 7b),[185,186] which provided important resources to identify key regulatory elements in cancer. The activity of these DNA elements classified cancer subtypes while identifying transcriptional regulators and gene regulatory elements involved in neoplastic expression control. This analysis reveals potential molecular pathways underlying cancer-predisposing inherited variants and tumorigenic somatic mutations in the regulatory genome.

4.3.2. Single-Cell Chromatin Accessibility Sequencing in Cancer Diagnostics. Single-cell chromatin accessibility sequencing enables the delineation of cell type-specific chromatin state. ScDNase-seq was applied to pools of thyroid cancer and normal cells, enabling the detection of thousands of tumor-specific DNase I hypersensitive sites (DHSs) in single cells.[130] DHS mapping revealed a tumor-specific mutation (chr18:52417839G > C) disrupting p53 binding and TXNL1 expression in follicular thyroid carcinoma. Droplet-based scATAC-seq mapped the chromatin landscapes of over 200,000 individual cells from human blood and basal cell carcinoma specimens.[133] scATAC-seq was also used to map cell type-specific chromatin accessibility landscape in the kidney and identified previously unrecognized cellular origin within heterogeneous papillary renal cell carcinoma (RCC).[187]

Another application of single-cell chromatin accessibility analysis is the creation of cell-type atlases in the human genome. scATAC-seq was used to generate chromatin accessibility landscapes in eight TCGA-classified tumor types at single-cell resolution (Figure 7c).[188] In another study, sci-ATAC-seq was used to map chromatin openness across 30 distinct human tissues, revealing chromatin accessibility patterns for >1.3 million single cells spanning throughout ontogeny, while mapping 1,154,611 cCREs across 222 cell types, covering 14.8% of the genome.[189] The cCREs atlas enables precise mapping of disease-associated noncoding variants to specific cell types and target genes for 240 complex disorders, revealing pathogenic cell populations and nominating cell-type-specific therapeutic opportunities. The Human Tumor Atlas Network (HTAN) seeks to create 3D atlases of three key transitional phases in cancer development. A technology combining scATAC-seq and RNA-seq contributed to the HTAN (Figure 7d),[190] mapping tumor evolution in both spatial and temporal dimensions at single-cell resolution. These transitions are driven by intricate crosstalk among premalignant, malignant, and/or nonmalignant cells in the tumor's cellular landscape. Technologies combining scRNA-seq and scATAC-seq constructed a detailed cancer-cell atlas comprising 54,971 single cells and delineated unique cellular subpopulations.[191] The data defined the key genes, cell types, and molecular pathways sustaining cancer stem cells and epithelial-mesenchymal transition in bladder cancer recurrence.

Single-cell multiomics integration enables the establishment of developmental epigenetic reference maps, facilitating the identification of conserved versus tumor-specific molecular features in clinical samples. Single-cell multiomic analysis discovers novel cell types and identifies biomolecular markers associated with metastatic heterogeneity. Combined scRNA-seq and scATAC-seq uncovered a novel cell subpopulation and identified CXCL14 as a key critical mediator of nodal metastasis in breast cancer.[192] Multiomics also revealed dynamic shifts in cellular composition and phenotypic state throughout the pathological progression from normal colonic epithelium to precancerous adenomas and ultimately to CRC. A significant proportion of cells in both adenomatous polyps and CRCs exhibited stem-like phenotypes, demonstrating progressive epigenetic remodeling along the transformation trajectory from normal mucosa to malignant tumors. Cancerous tissues showed T cell exhaustion, RUNX1-regulated cancer-associated fibroblasts, and increasing chromatin opening related to HNF4A motifs in epithelia.[193] Inverse-DNA methylation accessibility patterns in sporadic CRC enable molecular stratification of polyps.

4.3.3. Spatial Chromatin Accessibility Sequencing in Cancer Diagnostics. Spatial-ATAC-seq delineates anatomically restricted epigenetic landscapes and maps regulatory networks. Spatial-ATAC data can characterize the spatial organization of distinct cellular types and functional states, and the arealization of intricate tissue.[142] To characterize the chromatin landscape of diffusely localized or rare tissue cell populations in human lung parenchyma samples, LCM-ATAC-seq was used to test the myeloid cell marker PU.1. Tissues sections were initially immunostained for PU.1, with subsequent isolation and processing exclusively performed on marker-positive nuclei.[141] The findings established LCM-ATAC-seq to isolate highly enriched PU.1+ populations and characterize their chromatin accessibility landscapes. LCM-

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ATAC-seq allows spatial mapping of spatial chromatin landscapes in rare cell populations identified by specific marker genes within preserved tissue architecture. LCM-ATAC-seq is applied to different tissues and explores various targeting approaches, highlighting its potential for versatile use in diverse settings, such as tumor biology. Advanced spatial ATAC-RNA-seq coprofiling can reveal how chromatin accessibility regulates gene expression profiles and cellar dynamics with spatial resolution and whole-genome coverage. This approach offers key findings about spatial epigenetic initiation, differentiation, and regulatory networks in tissue microenvironments.[152] Spatial ATAC-RNA-seq joint profiles chromatin accessibility and gene expression on the same tissue section, identifying unique signatures and implicating their specialized functions in establishing cellular phenotypes.[152] Integrating epigenome and transcriptome data at single-cell resolution enables novel discoveries about spatial epigenetic priming, cellular differentiation, and gene regulatory networks in intact tissue architecture. In the near future, spatial technologies will provide detailed data on cell types and states, tumor boundaries, adjacent tumor microenvironments, and cell locations within cancer tissue.

5. CONCLUSIONS AND FUTURE DIRECTIONS

A key aspect of precision oncology is clinical stratification based on molecular biomarkers. Cancer progression is heavily influenced by nongenetic factors, with epigenetics serving as a critical layer of biomarker information. The field of epigenomic sequencing for cancer diagnostics is rapidly expanding, encompassing bulk, single-cell, spatial, and multiomic approaches to uncover various dimensions and multiple layers of information. Currently, bulk epigenetic biomarkers have been successfully implemented in clinical oncology for diagnostic and prognostic applications,[194−196] and several DNA methylation biomarkers demonstrate potential clinical results in the early detection of CTCs from patients.[197,198] Bulk sequencing holds promise in liquid biopsies for noninvasive cancer screening, especially detecting extracellular or cfDNA in bodily fluids for diagnostic information. For instance, detecting methylation patterns in potential cancer biomarker genes from peripheral blood samples devoid of malignant cells.[199] However, the major drawback of bulk-based epigenomic clinical applications stems from the unsolved problem of tumor heterogeneity. The averaged results from bulk sequencing may potentially mask rare but clinically significant subclones that are crucial for the diagnosis and predicting outcomes in highly heterogeneous cancers like glioblastoma.

Single-cell sequencing deciphers information not only on subclones, cell−cell interactions, and heterogeneous cell clusters but also offers a powerful tool for studying rare cellular subsets, including incipient primary tumors or tiny metastatic ones. Single-cell sequencing is valuable for analyzing low levels of genetic material from tumors in bodily fluids, such as CTCs. Additionally, multiomics technologies at single-cell resolution enable the systematic map of intricate cross-regulatory interactions among multiple epigenome layers with precise molecular resolution.

With the emergence of spatial omics technology, biomarkers derived from bodily fluids at bulk or tissue biopsies at single-cell levels will no longer suffice to meet the demand of translating clinical research. Spatial technologies generate high-dimensional data on cell types and states, cell boundaries, and cell locations within tissue. This data allows for the in-silico construction of tissue phantoms construction, providing a framework to elucidate clinical disease pathogenesis. It offers an effective platform for discovering tissue spatial biomarkers and provides a verification platform to identify prognostic epigenetic markers in oncology patients.

Although epigenomic technologies have been used clinically for cancer diagnosis, this field is relatively young, and many barriers must be overcome to operationalize its diagnostics application. Clinical adoption of these advanced tools remains constrained by high cost, stability, technical variability, reproducibility, rationality, practicality, accessibility, etc. Future technological advancement should focus on higher precision and multidimensional profiling with better accessibility. Evolving methodological breakthroughs to increase the sensitivity and specificity are essential to enhance the signal capture efficiency and reduce the technical noise and sparsity. A relatively small region of interest (ROI) limits the application of complete profiling in human samples. This is particularly true for spatial omics, where the number of input tissue samples is limited per profiling, making it challenging to fully capture the large-scale architecture of an organ. Techniques that integrate epigenomics with other omics, such as genomics, proteomics, and transcriptomics in cells or tissues, still require further development, and the associated bioinformatics tools need continued refinement.

Most current techniques perform optimally on fresh samples. However, FFPE-archived samples better preserve the hallmarks and morphology of cancer cells and tissues. Enhancing epigenomics technologies to tailor these samples could transform biomarker discovery into actionable clinical application. Additionally, integrating AI algorithms with H&E characteristics, tissue architecture spatially mapped molecular features, and gene expression will further revolutionize pathological evaluation capabilities. Integrating single-cell atlas data, high-resolution imaging, and clinical phenotype through multimodal AI frameworks enables the unlocking synergistic benefits for cancer diagnosis, facilitating the adoption of precision diagnostics in clinical application. In summary, ongoing improvements and advancements in epigenomics technologies will facilitate the integration of diagnostic strategies with multidimensional omics atlases across temporal scales, advancing our fight against human cancer.

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Author Contributions

L.L. wrote the initial manuscript with the supervision of L.Y. L.L. and L.Y. revised and finalized the manuscript.

Notes

The authors declare no competing financial interest.

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■ VOCABULARY

Epigenomics: Omics that large-scale study the epigenetic modifications, such as DNA methylation, histone modifications, and chromatin accessibility, which regulate gene activity without changing the DNA sequence.

DNA methylation: It is a key epigenetic modification where a methyl group ($-CH_3$) is added to the cytosine base in DNA, typically at CpG sites where cytosine is followed by guanine.

Histone modifications: These modifications, such as acetylation, methylation, phosphorylation, and ubiquitination, are chemical changes to histone proteins (around which DNA is wrapped) that alter chromatin structure and act as ″epigenetic marks″ that influence whether genes are turned on or off.

Chromatin accessibility: It describes how ″open″ or ″closed″ chromatin is at a given genomic region, determining how easily regulatory proteins such as transcription factors, enzymes, or other regulatory proteins can access the DNA to control gene expression.

NGS technology: This technology refers to advanced, high-throughput DNA/RNA sequencing methods that allow rapid and cost-effective analysis of entire genomes, transcriptomes, or targeted regions.

Cancer diagnostics: The methods and technologies used to detect, characterize, and monitor cancer in patients. It involves identifying cancerous cells, determining the tumor type, stage, and genetic/molecular profile to guide treatment decisions.

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