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Shedding light on DNA methylation and its clinical implications: the impact of long-read-based nanopore technology

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Abstract

DNA methylation is an essential epigenetic mechanism for regulation of gene expression, through which many physiological (X-chromosome inactivation, genetic imprinting, chromatin structure and miRNA regulation, genome defense, silencing of transposable elements) and pathological processes (cancer and repetitive sequences-associated diseases) are regulated. Nanopore sequencing has emerged as a novel technique that can analyze long strands of DNA (long-read sequencing) without chemically treating the DNA. Interestingly, nanopore sequencing can also extract epigenetic status of the nucleotides (including both 5-Methylcytosine and 5-hydroxyMethylcytosine), and a large variety of bioinformatic tools have been developed for improving its detection properties. Out of all genomic regions, long read sequencing provides advantages in studying repetitive elements, which are difficult to characterize through other sequencing methods. Transposable elements are repetitive regions of the genome that are silenced and usually display high levels of DNA methylation. Their demethylation and activation have been observed in many cancers. Due to their repetitive nature, it is challenging to accurately estimate DNA methylation levels within transposable elements using short sequencing technologies. The advantage to sequence native DNA (without PCR amplification biases or harsh bisulfite treatment) and long and ultra long reads coupled with epigenetic states of the DNA allows to accurately estimate DNA methylation levels in transposable elements. This is a big step forward for epigenomic studies, and unsolved questions regarding gene expression and transposable elements silencing through DNA methylation can now be answered.

Keywords Nanopore sequencing, Long-read sequencing, DNA methylation, Epigenomics, Methylome

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Introduction

The Human Genome project started in 1990 and it was completed in 2004 (the first draft of the genome was submitted in 2001) [1–3], paving the way for the 1000 Genomes Project, which was initiated in 2007 with the aim of sequencing 1000 genomes. Its final phase was completed in 2015, when a very large amount of data was structured in an open-access database of genomic information from 2504 participants [4, 5]. Whole-genome sequencing was, therefore, a first step in uncovering previously unknown information regarding the DNA (deoxyribonucleic acid), and it has paved the way for epigenomics and sequencing whole methylomes.

DNA methylation is the process of adding methyl groups to the fifth carbon of cytidine (C5) or in the N6 position of adenine within the DNA strands, and it is considered the most frequent and stable epigenetic modification associated with silencing of gene transcription [6–8]. DNA methylation is an important epigenetic mechanism as it can regulate gene expression through inhibition of transcription factor binding to DNA. *De novo* DNA methylation and demethylation both occur in a dynamic manner during development, creating unique DNA methylation patterns which are specific to every cell. This process contributes to cell differentiation and tissue-specific gene transcription. Nevertheless, recent studies have shown that the relationship between DNA methylation and gene expression is more complex and addition of DNA methylation to gene promoters results in transcription silencing only in a relatively small subset of genes [9]. Supporting this, another recent study showed that systematic differences in DNA methylation between males and females on autosomal chromosomes do not correlate with differences in gene transcription [10]. However, only large variations in DNA methylation at specific regulatory sites (5'UTR and promoters), do indeed display correlation with variation in gene expression [11].

While proper functioning of DNA methylation is crucial for genomic imprinting, X chromosome inactivation, or silencing of retroviral elements [12], dysregulation can be responsible for malignancy (both solid and hematological cancer) and other diseases [13–15].

DNA methylation was first discovered as the presence of 5-methylcytosine (5mC) in the DNA of *Mycobacterium tuberculosis* in 1925, by Johnson and Coghill [16, 17]. After a hiatus of 23 years, Hotchkiss has described DNA methylation in calf thymus DNA in 1948 [18, 19]. At the end of the 1970s, it was generally accepted that DNA methylation has an important role in suppressing gene expression [16], and its role in cell differentiation has begun to crystallize after the publication of a study on undifferentiated murine embryonic cell lines treated with DNA methylation inhibitor, 5-azacytidine, in which new cell phenotypes had been formed [13, 20]. The concept of epigenetics proposed by Waddington in 1942 [21] has been refined by Riggs, who included DNA methylation as an epigenetic marker in 1996 [18, 22]. Currently, DNA methylation is the most studied mechanism of epigenetics and it is responsible for a wide range of effects on development, aging and disease [16].

In 1992, bisulfite sequencing emerged as a method to estimate methylation levels, and it quickly became the gold standard method for generating genome-scale methylation maps called methylomes [16, 23]. This method involves chemically treating DNA with sodium bisulfite (an invasive method that damages DNA strands), subsequent amplification with bisulfite-specific primers, followed by sequencing. Afterwards, next-generation sequencing was developed as a tool for detecting methylation in short DNA strands (short-read sequencing) [6, 16, 24].

Recently, long-read sequencing has become possible by overcoming read-length limitations through two new technologies that have been developed for genome-wide studies: single molecule real-time (SMRT) sequencing from Pacific Biosciences (PacBio) and nanopore sequencing from Oxford Nanopore Technologies (ONT) [6, 25]. Both technologies can be used for analyzing short strands of bisulfite-treated DNA just as the old techniques, but the main advantage is that bisulfite treatment is no longer required for sequencing DNA, as ONT and SMRT can detect many types of DNA modifications simultaneously on long strands, allowing genome, epigenome, transcriptome and epitranscriptome profiling without specific prior sample preparation [25–27].

Clinical applications of DNA methylation

One of the many important processes involved in epigenetics is the modification of DNA structure through adding chemical residues, such as methyl, carboxyl, hydroxymethyl, dimethyl and many others, to one of the four standard nucleotides: adenine (A), cytosine (C), guanine (G), thymine (T). The most studied type of such modifications is DNA methylation, which appears when a methyl group is added to C or A within the DNA [28].

During early embryogenesis, there is an epigenetic reprogramming consisting of the development of global DNA demethylation and remethylation [29], which indicates limited evidence for transgenerational inheritance of DNA methylation in mammalian systems [8, 30]. Nevertheless, it has been hypothesized that environmental exposures are able to modify the epigenetic control on gene expression, and that the phenotype is the result of the interaction between genotype and environment. This explains why the methylome has proved to be dynamic during development, cell differentiation and aging [30–32]. Identifying DNA methylation in the genome is very

important for understanding the purposes of epigenetic modifications [6].

Almost half of the human genome (~45%) consists of silenced viral and transposable elements, which display high levels of DNA methylation. Therefore, methylation is essential, as the expression of these harmful elements could lead to gene disruption and mutations [12]. Nevertheless, some of these TEs have been co-opted by the host to perform essential regulatory functions (e.g. TEs are upregulated during early development and in the neuronal lineage and dysregulations have been proved to be involved in the development of neurological disorders and cancer [33]).

The base pairing of cytosine and guanine occurs through phosphate (p) links, therefore the abbreviated form of C-G dinucleotides is CpG (cytosine-phosphate-guanine). CpGs do not have an even distribution throughout the genome, as they tend to assemble in their unmethylated form in areas called CpG islands (CGIs), which are usually associated with gene promoters [13]. The rest of the CpG sites found throughout the human genome (70–80%) are methylated, predominantly as 5mC [18], and they transform into thymine through deamination over time [34].

Apart from 5mC and 5hmC, there are other forms of methylation present throughout the human genome, such as N6-methyladenine (6 mA, involved in the development of different types of neoplasia, such as gastric and non-small-cell lung cancer [35, 36]), N4-methylcytosine (4mC, involved in the development of gastrointestinal cancer [37]), and the oxidation products 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC, possibly involved in the development of prostate cancer) [6, 18, 38].

The chemistry of DNA methylation: DNA methyltransferase enzymes

DNA methylation is possible due to DNA methyltransferase enzymes (DNMTs) which are able to transfer a methyl group from S-adenosyl methionine (SAM) to the fifth carbon of C within the DNA to form 5mC [12], fulfilling an essential role in embryogenesis [13, 39]. The first DNMT (DNMT1) was discovered in 1988 [40], followed by DNMT3a and DNMT3b in 1998 [41]. Recently, DNMT3L has been described as a cofactor, while in 2016, DNMT3C has been discovered as a new methyltransferase involved mostly in fertility by protecting male germinative cells from the activity of transposons [42]. These are not the only important enzymes involved in the methylation process. They can actually be categorized in 3 groups of enzymes: the writers, erasers, and readers. DNMTs are writers, catalyzing the addition of methyl to C residues. Erasers, such as the ten-eleven translocation (TET) enzymes [12] which have been discovered in 2010 [43], and the activation-induced cytidine deaminase (AID) / apolipoprotein B messenger ribonucleic acid (mRNA) editing enzyme, catalytic polypeptide (APOBEC), are responsible for demethylation, which consists of removing the methyl groups from DNA. Readers, such as methyl-CpG-binding domain (MBD) proteins, ubiquitin-like, containing PHD and RING finger domains (UHRF) proteins, and zinc-finger proteins, are able to bind methyl groups in order to influence the expression of genes [12].

DNMTs fulfill complementary roles in order to maintain the methylation patterns in mammals. DNMT1, DNMT3a, DNMT3b and DNMT3c have different, but essential purposes [18, 42] (see Fig. 1).

DNMT1 is known as 'the maintenance enzyme', which ensures that the methylation pattern is preserved between cell divisions. It is the best studied DNMT, and it is present in high concentrations in dividing cells, as it has an affinity for the hemimethylated DNA present at the replication fork during DNA replication. It has the ability to bind to the newly synthesized DNA strand and it adds methyl groups according to the prior pattern.

DNMT3a and DNMT3b are known as 'de novo methyltransferases', because they add methyl groups to unmethylated C within the DNA strands. They have a similar structure, as for the functions, DNMT3a tends to be ubiquitously expressed and it is important for cell differentiation and maternal imprinting, while DNMT3b has a low expression in differentiated tissues, except for bone marrow, testes and the thyroid gland, and it is crucial in early development and X-chromosome inactivation in females [12, 18]. DNMT3L has recently been discovered as a member of the DNMT family lacking a catalytic domain, which is able to stimulate the enzymatic activity of DNMT3a and DNMT3b, while being expressed mostly in early development [12, 44, 45].

Demethylation (mechanism of methyl groups removal) can occur passively through a loss of maintenance during cell division, or actively through eraser enzymes such as TET enzymes (TET1, TET2, TET3), which are able to transform 5mC groups into 5-hydroxymethylcytosine (5hmC) [13, 30]. Numerous 5hmC residues are present in the developed brain, and it is unclear if 5hmC is only an intermediate step in DNA demethylation or if it has its own epigenetic roles in gene expression [12, 46]. The inhibition of methylation enzymes (DNMTs) with different molecules such as azacitidine and decitabine (drugs used in oncology), can be useful in modifying the malignant phenotype of the cells through re-expression of tumor suppressor genes [8, 47, 48].

Roles of DNA methylation

The process of DNA methylation is essential for normal development in mammals, having important roles

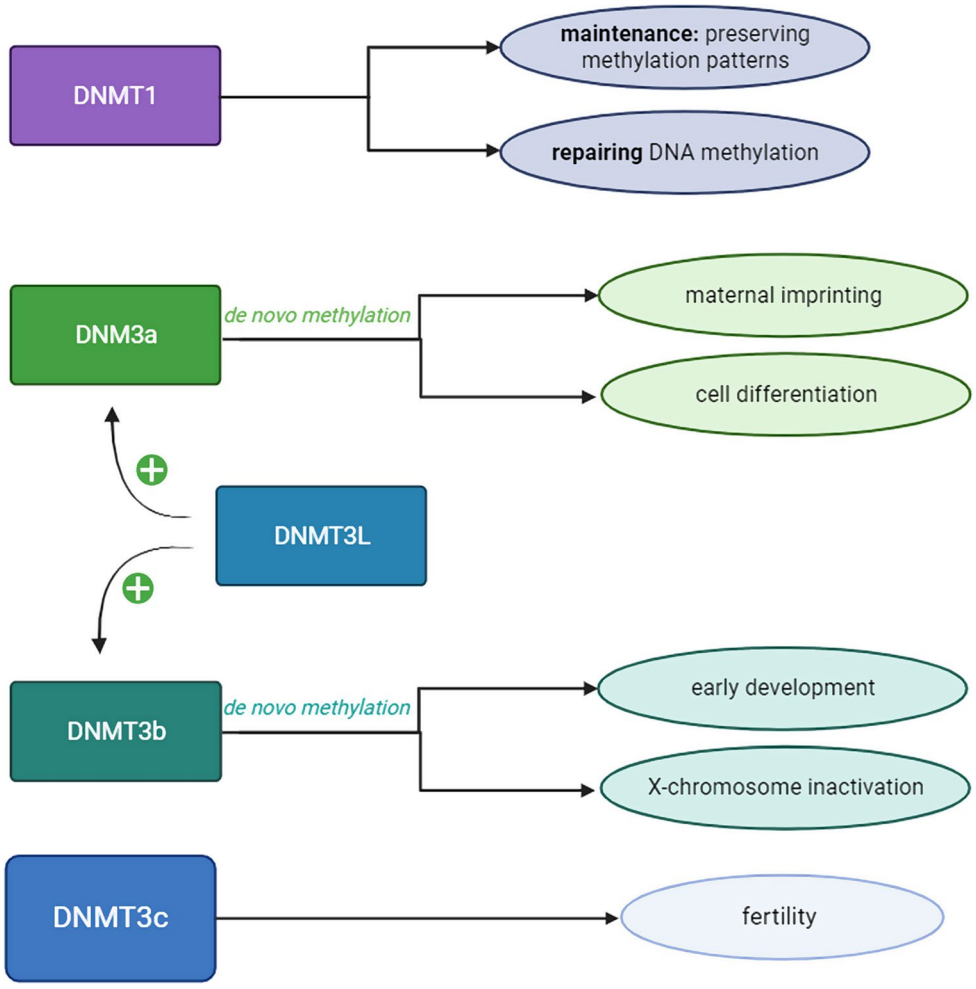

Fig. 1 The roles of DNA methyltransferase enzymes (DNMTs) [12, 18, 42]

in early embryonic development, stem cell differentiation, tissue maturation, and whole genome studies have proved that methylation is cell-type specific [30, 49]. However, cell-to-cell epigenetic variations have been identified within homogenous cell populations, therefore DNA methylation could be considered an important factor in biological variability of malignant tumors [6, 50].

Many physiopathological processes, both normal and abnormal, have been associated with DNA methylation (especially 5mC) (see Fig. 2): X chromosome inactivation, genomic imprinting, chromosome stability [18] and structure modulation, transposon activation, inflammation [6], genome integrity and stability, poly(A) tail length regulation [1, 51], silencing repetitive DNA [13]. Effects on RNA splicing, degradation and translation have been associated with the presence of 6mA [1]. Some of the pathological processes linked to methylation are: malignancy, imprinting disorders (Angelman syndrome, Prader-Willi syndrome, Beckwith-Wiedemann syndrome, Silver-Russell syndrome), X-chromosomal recessive disorders (e.g. Duchenne's muscular dystrophy, Haemophilia B), trinucleotide repeat disorders (e.g. Fragile X syndrome, Friedreich ataxia), defects in gene expression regulation machinery (e.g. Immunodeficiency, Centromere instability and Facial anomalies syndrome (ICF syndrome), Alpha thalassemia X-linked intellectual disability (ATRX syndrome), Rett syndrome) [8], as well as phenotypes of developmental delays and congenital anomalies which have yet been attributed a specific genetic cause [52].

Epimutations can be defined as random errors of the epigenetic machinery, which can be associated with a multitude of environmental factors (e.g. tobacco smoking, foods and dietary factors) that have effects on DNA methylation [8, 31].

X-chromosome inactivation

DNA methylation is responsible for the inactivation of one of the two X-chromosomes present in females [8], through high rates of methylation found in the proximity of promoter regions. Normally, in this area, the DNA tends to be unmethylated in order to allow the

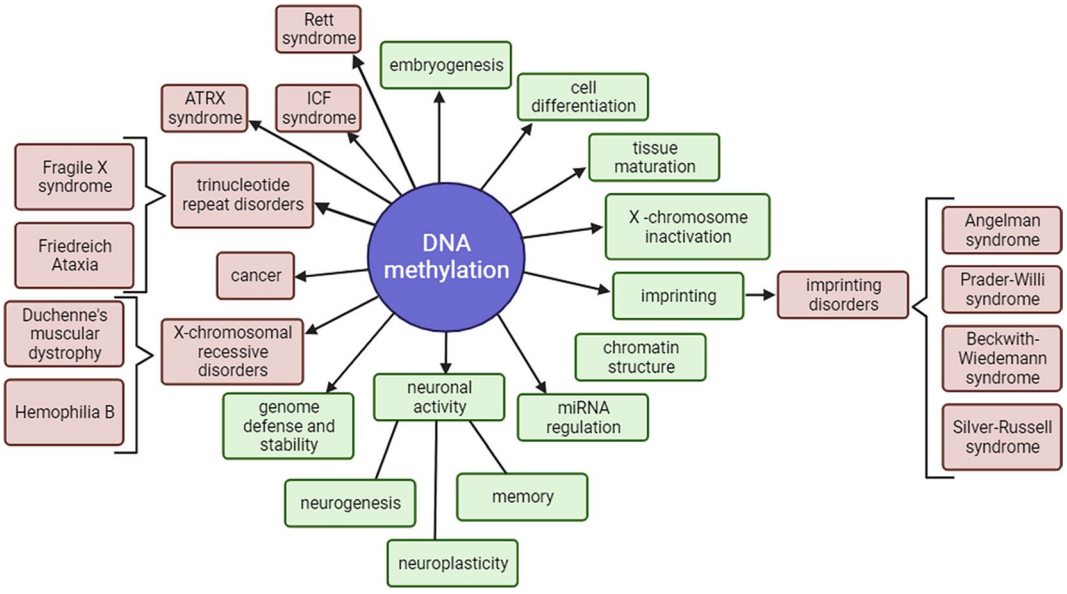

Fig. 2 Physiopathological processes in which DNA methylation is involved. The physiological processes are colored in green, while the pathologies are colored in red. Abbreviations: Immunodeficiency, centromere instability and facial anomalies syndrome (ICF syndrome), Alpha thalassemia X-linked intellectual disability (ATRX syndrome)

actions of gene promoters; however, when DNA methylation occurs, the genes are silenced and the respective X-chromosome is inactivated [18]. This process is coordinated in early development by the long non-coding RNA (lncRNA) XIST, which is first transcribed and afterwards it spreads in *cis* across the inactive X-chromosome. It has been thought that this is the only role of XIST, but recently it has been discovered that XIST is still needed in adult human B-cells for silencing X-linked immune genes (e.g. TLR7) through XIST-dependent histone deacetylation, since these genes lack promoter DNA methylation [53].

Imprinting

Recently, DNA methylation analysis from blood samples has become the first diagnostic procedure in the management of patients with a suspicion of imprinting disorders [8]. Gene regulation through methylation of CGIs is an important step for imprinting [12]. Usually, most gene transcripts are expressed from both parental alleles: maternal and paternal. Imprinted genes are genes expressed only from the maternal or the paternal allele, based on imprinting control regions: these are parent-of-origin (PofO) differentially methylated regions (DMRs) [7]. There are hundreds of differentially methylated loci in the human genome, according to the PofO, which are quite constant across tissues, individuals and populations. The differential methylation occurs in gametes or after fertilization, and it persists in adults [54], fulfilling essential roles in both imprinting, and embryogenesis [55]. DMRs can also have crucial roles in determining phenotypes (e.g. altering the expression of mismatch repair genes could lead to malignant growth) [49].

Allele-specific methylation patterns are difficult to identify through short-read sequencing. In 2012, Fang F. et al. have developed a probabilistic model, independent of genotype, in order to identify allele-specific methylation patterns based on data obtained from bisulfite sequencing, by describing how the methylation state of each read reflects two distinct patterns which contain half the data. This was a first step in integrating computational strategies in order to enhance the accuracy of detecting allele-specific methylation patterns [56]. In 2015, progress was made in this specific area through a method called Pyrosequencing, a real-time sequencing method which was able to analyze the patterns of methylation separately on each allele. It was able to identify individuals heterozygous for a SNP from a region of interest, and the bisulfite-treated DNA was then analyzed for identifying regions of potential allele-specific DNA methylation surrounding that specific SNP. The newly discovered DNA methylation patterns were then individually amplified using allele-specific PCR in order to be further analyzed in a more detailed matter [57]. Currently, an easier and more efficient method for identifying allele-specific methylation patterns is long-read sequencing, through nanopore technology [7, 49].

Alterations of imprinting are responsible for many diseases: Prader-Willi Syndrome, Angelman Syndrome, Beckwith-Wiedemann Syndrome, and cancer [55], and the diagnosis for known imprinting disorders can be made through simultaneous screening [58]. Prader-Willi

syndrome appears through loss of the paternal allele of chromosome 15q11-q13 [8]and it is characterized by hypotonia in infants, followed by obesity and excessive eating after early childhood, associated with significant mental impairments [12, 58]. The loss of the maternal allele of chromosome 15q11-q13 causes Angelman syndrome [88], associated with epilepsy, intellectual disability, limited speech and truncal ataxia [58]. Silver-Russell-syndrome occurs through a somatic mosaic defect and it is characterized by severe intrauterine and postnatal growth impairments. The same mechanism applies to Beckwith-Wiedemann syndrome, which is associated with overgrowth, malformations and predisposition to tumors [8, 58].

Repetitive sequences-associated diseases

Transposable elements (TEs) are DNA elements that change chromosomal position and copy number within a genome. There are two main classes of TEs, namely: (i) DNA transposons that use a cut-and-paste mechanism and (ii) retrotransposons that replicate their DNA copies through an RNA intermediate. Integration of TEs either through cut-and-paste or retrotransposition can happen either inside genes (affecting the sequence of the gene) or in the vicinity of genes (which can lead to abnormal activation or repression of the neighboring genes) [59]. Transposable elements can be directly associated with a number of diseases, such as hemophilia (which could be considered as the prototype for a disease-causing insertion), muscular dystrophy, various types of cancer [60] (cancers of the pancreas, colon, ovaries [59], breast [61], bladder [62], liver [63], B-cell non-Hodgkin lymphoma [64]), senescence of mesenchymal progenitor cells [65], amyotropic lateral sclerosis [61], Alzheimer's disease, Parkinson's disease, Huntington's disease, Friedreich ataxia, Fragile X Syndrome, Fronto-temporal lobar degeneration, multiple sclerosis, Aicardi–Goutières syndrome, Autism spectrum disorders, mental disorders (schizophrenia, bipolar disorder, major depressive disorder, post-traumatic stress disorder), Creutzfeldt–Jakob disease, neurofibromatosis type I [66].

The silencing of TEs is important in the maintenance of genome integrity and correct gene regulatory programmes. Thus, they are heavily methylated, but hypomethylation of repetitive sequences might appear due to aging or malignancy [13]. In particular, epigenetic reactivation of cryptic regulatory elements within TEs is responsible for oncogenesis, and this process is known as onco-exaptation [67]. In many types of cancer (e.g. pancreatic, colonic, ovarian cancer and glioblastoma), hypomethylation of the L1 promoter (which is a very well known TE) is present and it is able to exert an influence on histologic grade, clinical stage and overall survival [59].

Other pathologies, such as Huntington's disease, Friedreich ataxia and Fragile X Syndrome, are a few of the diseases associated with errors in the epigenetic regulation of repeat expansions, not necessarily related to TEs [25, 68].

Cancer

The most frequent epigenetic alteration in cancer is DNA modification of 5mC, and abnormal methylation can also be associated with resistance to oncologic therapy [69], which is why DNA methylation biomarkers serve as an important tool for early detection and cancer treatment [70]. Demethylation agents are currently used as an important type of cancer treatment [8]. However, both hypermethylation and hypomethylation can become mechanisms in the development of cancer.

In malignancy, tumor suppressor genes are silenced, therefore the most commonly known epigenetic aberration found in cancers is the hypermethylation of promoters which is responsible for silencing certain genes. The promoters of numerous genes involved in DNA repair and regulation of the cell-cycle can be targeted and involved in aberrant DNA hypermethylation, leading to genomic instability [8, 18, 71]. For example, silencing of p16INK4 due to DNA methylation promotes abnormal cell proliferation through increased levels of retinoblastoma protein (pRB), being involved in the etiopathogenesis of hepatic, lung, pancreatic, breast, cervical and bladder cancers [14, 15, 72, 73]. Hypermethylation of BRCA1 has been discovered in ovarian and breast cancers [74].

Hypomethylation is usually present in the early stages of cancer as it promotes malignant cell transformation and genomic instability, while also being able to activate silenced genes. For example, melanoma-associated antigen A (MAGEA), which belongs to a class of cancer-testis antigens whose promoters are known to be methylated in healthy tissues (except for testis), is initially silenced through hypermethylation, but its promoters can be activated through demethylation, leading to increased tumor aggressiveness in lung, breast and colorectal cancers [13, 75, 76].

DNA methylation analysis is currently done in medical practice for identifying epigenomic alterations in SEPT9, MGMT, MLH1, SHOX2 [88]. Future perspectives could bring methods which are able to simultaneously analyze genomic and epigenomic alterations in cancer through nanopore whole-genome sequencing. Magi et al. have conducted such a study on patients with acute myeloid leukemia [69].

As it has been previously stated, DNMTs and TETs are essential for the methylation of DNA, but they fulfill important roles for normal hematopoiesis as well. Therefore, mutations involving these enzymes have been

associated with the development of hematological malignancies. DNMT3A mutations are clearly associated with acute myeloid leukemia (AML) [77], and their presence was noted in 22% of the cases of AML. There has also been hypothesized that DNMT1 mutations could have a role in developing AML, as an increased sensitivity to DNMT1 mutations was observed in some DNMT3A mutant cellular models, but a clear conclusion has yet to be made [78]. While speaking about TETs, the most frequent mutations associated with clonal hematopoiesis, myelodysplastic syndrome (MDS), and AML were specifically loss-of-function mutations in TET2, through DNA hypermethylation phenotypes [79]. Reducing the function of TET3 through hypermethylation, however, could be also potentially involved in the development of AML alongside TET2 mutations [80].

Circulating cell-free DNA (cfDNA) is also a promising biomarker which can be detected in blood, stool or urine samples for cancer screening and estimating the therapeutic response, as well as identifying collateral damage in the tissues found in the proximity of the tumor, since DNA methylation can differentiate between malignant and non-malignant cells [70, 81]. However, detecting cfDNA raises a few challenges due to the molecular heterogeneity of cancer, sample sizes and low fractions of cfDNA. Stackpole et al. have developed cfMethyl-Seq as a cost-effective sequencing tool for cfDNA methylome, but the method needs to be tested further before introducing it as a standard procedure [82].

Chromatin structure

DNA methylation is involved in modifying and shaping the structure of chromatin, which ultimately translates as an important role in maintaining genome stability and regulating gene expression [18]. DNA is normally tightly 'packed' in structures called nucleosomes, which involve the wrapping of DNA strands around proteins called histones - the tighter the DNA is packed, the more difficult it is for genes to be expressed. CGIs contain less nucleosomes, therefore allowing a higher gene expression than other parts of the genome. The role of DNA methylation in this process relates to modifying the structure of histones, usually promoting gene repression [12, 83].

Non-coding RNAs regulation

Micro-ribonucleic acids (miRNAs), a type of non-coding RNAs, are biomolecules involved in the regulation of gene expression, representing a newly discovered epigenetic mechanism which can be studied through nanopore technology [84]. The fact that methylation is responsible for regulating the expression of miRNAs was discovered through a study on a colonic cancer cell line, where the loss of DNMTs was able to reactivate silenced miRNAs, promoting tumor growth [12, 85].

Neuronal activity

DNA methylation appears to have a dynamic nature in neurons, as it can be regulated by physiological or environmental (drugs, injuries, electroconvulsive stimulation, exercise) factors, which ultimately influence neuronal activity and cognitive functions. A murine study has shown that apart from the increased methylation levels occurring in CpG sites, there are also non-CpG sites showing high methylation, but the purpose of non-CpG methylation has yet to be discovered [12, 55].

DNA methylation also fulfills important roles in neurogenesis, cell differentiation, neuronal maturation, neuroplasticity, learning and memory, while it is also present and could potentially be involved in the pathogenesis of schizophrenia bipolar disorder [12]. Weaver et al. have conducted a study which eloquently illustrates the influence of methylation in brain development: maternal neglect classified as a form of early-life stress has been linked to increased DNA methylation in the brain of murine models, which has been conserved through adulthood especially in promoters of the glucocorticoid receptors, therefore altering the response to stress (which was heightened in neglected murines) [86].

Detection of DNA methylation

Many laboratory techniques can be used for detecting DNA methylation, but one initial setback was that DNA methylation is not replicated and amplified through polymerase chain-reaction (PCR). Three categories of methylation assays have been classically described: (1) techniques involving antibodies against methylated C, (2) bisulfite treatment of unmethylated C, (3) restriction enzymes which can cut DNA in the presence/absence of methylation [8, 87]. First, second, third and even fourth generation sequencing techniques have been described.

Bisulfite sequencing and enzymatic methyl sequencing

Bisulfite sequencing is able to detect methylated C through a chemical process which transforms unmethylated C in uracil (U), which can be amplified as T through PCR [13, 71]. Bisulfite sequencing is still currently the gold standard method for detecting DNA methylation in patients with cancer [8].

The chemical reactions which occur in the bisulfite sequencing protocol result in altering and degrading the DNA, providing only short strands for analysis, and therefore not being used for long-read sequencing [71]. DNA degradation through this harsh treatment involving extreme temperatures and pH is the major disadvantage of this sequencing technique, but there are also another setbacks such as low specificity, low DNA sequence diversity through the short fragments and difficult haplotyping (because short reads have a smaller probability of

containing single-nucleotide polymorphisms (SNP) than longer strands) [17, 42].

Taking all these issues into consideration, a different method which would not fragment the DNA was necessary. Therefore, enzymatic methyl-seq (EM-seq) was developed as a means of identifying 5mC and 5hmC while keeping the structure of DNA. This method involves three enzymes (TET2, T4-BGT and APOBEC3A) and two chemical reactions: firstly, DNA is treated with TET2 and T4-BGT so that 5mC and 5hmC could not be deaminated, and secondly, APOBEC3A is used for deaminating the remaining unmodified cytosines [89].

Next generation sequencing

In long-read sequencing, multiple nucleotide sequences can be read at once, without the prior need of DNA cleavage and amplification [13]. This provides a great premise for reconstructing haplotyped methylomes, for allowing insights on allele-specific 5mC patterns and their effects on mutations and gene regulation, while also being able to provide epigenetic information about repeat-rich regions [25, 30, 49]. Third generation sequencing is performed through single-molecule real-time (SMRT) sequencing provided by PacBio, which is able to analyze the kinetics of polymerase [49], fulfilling an impactful role in genetic disorder diagnosis, especially for repetitive sequences-associated diseases, where short-read sequencing is not able to provide correct diagnoses through technical limitations (the length of the reads could not be sufficient for mapping repetitive regions longer than the respective read) [52].

Nanopore sequencing

Long-read sequencing (LRS) works with strands of DNA starting from 5 to 10 kilobases up to several megabases (compared to second generation sequencing which worked with 300–400 bp length strands of DNA), providing great promise in the fields of omic studies such as genomics, epigenomics and transcriptomics. The major advantages of LRS compared to short-read NGS include: improved structural variation detection, better resolutions for highly repetitive regions, the ability to detect DNA modifications directly from sequencing data, and an accurate long-range haplotype phasing [52]. Genome-wide methylation profiling, which can analyze the methylation status of each CpG site within the genome, is a powerful tool for identifying the influence of environmental factors on the methylation of DNA through LRS [13, 31].

The concept of using nanopore sensors for sequencing DNA was hypothesized in the 1980s through the identification of α-hemolysin as a nanopore able to recognize ionic current blockades [90], and currently nanopore sequencing developed by Oxford Nanopore Technologies (ONT) represents the fourth-generation sequencing technique which is able to rapidly analyze long sequences of DNA without the prior need of PCR amplification [13], as well as to analyze DNA, RNA [84], or even drugs, polymers and macromolecules [91] (see Fig. 3).

ONT sequencing and the SMRT sequencing technique provided by PacBio are currently the two main methods used for LRS. LRS itself is able to detect 3% more CpGs than the short-read sequencing methods, due to its ability to accurately capture challenging regions. ONT and PacBio sequencing technologies show some differences in the fields of error rate, throughput and the length of the generated reads. Sigurpalsdottir BD et al. have conducted a comparative study that has concluded that ONT is able to generate longer reads with lower sequencing costs per sample, but this technology was associated with a higher error rate than PacBio. CpG methylation detection through ONT sequencing, however, has proven to be highly accurate despite the slightly higher error rates, showing similar results to SMRT PacBio [92].

In 2014, a pocket-size nanopore sequencer known as MinION, developed by ONT [90], was approved for commercial purposes and it has been used for viral genome sequencing in the Ebola and Zika virus outbreaks [93]. MinION measures the ionic current variations caused by the passing of a single-stranded nucleic acid from a blood or saliva sample through a nanopore [25, 93].

Nanopores, which have a diameter of approximately 10^{-9} m, serve as biosensors enclosed in an electrically resistant polymer membrane, to which a constant voltage is applied within an electrolytic solution (electrophoresis). Through this method, an ionic current is produced, and long DNA or RNA strands can pass through the nanopore due to their electric charge, from the negatively charged 'cis' side to the positively charged 'trans' side. Motor proteins are used for controlling the translocation speed, improving the signal-to-noise ratio. These are processive enzymes (e.g. phi29) that are able to slow the translocation of DNA through the nanopore [13, 90].

The transmitted electrical signals are sensitive to DNA modifications (which can deviate the raw signal), which is why this laboratory technique can be used for differentiating methylated from unmethylated cytosine residues in the DNA molecule [24]. The current trace is translated into nucleotides through basecalling, a crucial process for detecting DNA base modifications, followed by anchoring the raw signal to genomic references and reviewing the evidence of the base modification. By using different computational tools, nanopore sequencing is able to identify sequence bases, DNA/RNA modifications, or to predict the length of the poly(A) tail and the secondary structures of RNA [25, 27].

A few examples of nanopore sequencing devices are: MinION (512 channels, with 4 nanopores/channel),

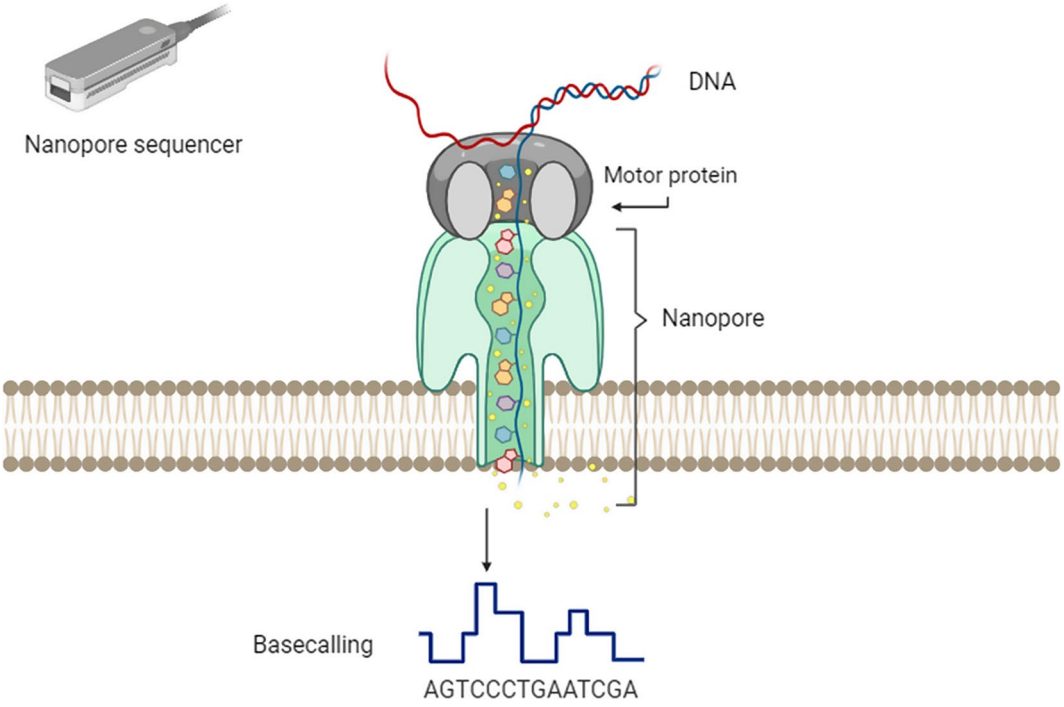

Fig. 3 The principle of nanopore sequencing. The DNA strands pass through a biological or synthetic nanopore that can have an enzyme motor protein attached, generating an ionic current. The motor protein is able to control translocation speed through the nanopore. The ionic current is afterwards translated into nucleotides through the process of basecalling. Created with http://BioRender.com. Adapted from references [1, 13, 27, 84, 90]

GridION, PromethION (for large scale sequencing), Flongle (a flow cell adapter of only 126 channels), SmidgION (a smartphone-compatible device, first announced in 2016 and currently still in development) [90]. The first commercialized ONT device was MinION in 2014, which is small and portable (10×3×2 cm, 90 g) [94], serving as an important tool for in-the-field sequencing [95], and can generate up to 30 GB of DNA data [6]. For analyzing the data generated by MinION, a computer with at least Windows 7 or Mac, with a solid-state drive (SSD), more than 8 GB of RAM and more than 128 GB of hard disk space is needed, as it has to operate the MinKNOW software. Many other applications have been developed for different purposes, such as genome assembly or variation detection, but MinION is not the ideal tool for large genome analyses due to its low throughput [94]. Medium throughput (GridION, 2017) and high throughput devices (PromethION, 2018) have emerged afterwards for improving the accuracy of DNA sequencing [6, 52]. The nanopores have been continuously updated and refined, and there are currently 9 system versions versions [90], with the latest being R10.4.1 (2022), which reports a 99% single molecule accuracy [52].

The main **advantages** of nanopore sequencing are: small size (the devices are portable and usable in the field), no prior need of DNA amplification, no apparent limit of DNA strand length [95] (ultra long reads, showing variations within the whole genome), high throughput [91], no bias in sequencing quality with read length (useful in analyzing highly repetitive regions of the DNA) [49], distinguishing 5hmC signals from 5mC signals and allele-specific methylation [6], low prices (and currently going down, becoming comparable to those of short-read sequencing through bisulfite techniques) [25, 71, 96], fast sample preparation and analysis (the full process of complete methylation analysis only takes a few hours, and depending on the GPU power of the computer that is used it could sometimes take only 1–3 h, which is promising for real-time applications in cancer) [81], high accuracy ranging from 96.3–98.8% [97].

Nanopore sequencing also comes up with a few **disadvantages**: limited discrimination between heterozygous and homozygous alleles [98], long and rare methylation motifs can be missed by nanopore sequencing [99], the need for high-quality DNA of high molecular weight (requiring invasive techniques for extraction from blood or other tissues) [52].

Future perspectives for the use of nanopore sequencing include in-detail analyses of telomeres and the relationship between their length and environmental factors [84, 100], liquid biopsies [81], intraoperative neuropathological classifications [93], direct detection of modified nucleobases incorporated in recently replicated DNA, which could be useful for mapping genome-wide replication at the single-molecule level [101].

Nanopore sequencing in methylation studies

Nanopore sequencing is useful for studying DNA methylation on long strands of DNA, as well as for identifying repetitive elements, which are difficult to characterize through other sequencing methods [93]. The practicality of nanopore technology has been proven in 2013, when it was discovered that it can distinguish methylated cytosines from native cytosine through the characteristics of the current signals. Since then, several bioinformatic tools have been developed for identifying 5mC (Nanopolish; through a HMM, 5mC modifications can be detected in *Escherichia coli* and humans, but non-CpG methylations could not be detected), 5hmC (signalAlign; used for *Escherichia coli* DNA) and 6 mA (signalAlign and mCaller; the latter can detect 6 mA modifications in *Escherichia coli*, mice and Lambda phage DNA) in the genome, which have proved to be useful in methylome characterizations of bacteria (*Escherichia coli*, *Chlamydomonas reinhardtii*) and humans [28, 90]. Long-read nanopore sequencing is able to generate haplotyped mammalian methylomes, meaning that the level of CpG methylation in the DNA and the haplotype from which the reads arise can be detected [49]. The epigenetic profiling of *Drosophila melanogaster* genome, which possesses 6 mA modifications responsible for crucial functions across many tissues throughout development [102], human transposons and X-chromosome telomere-to-telomere assembly also became possible through nanopore sequencing [27]. There has also been a focus on combining this technique with other methods for enhanced epigenomics profiling: nanopore Cas9-targeted sequencing was used for assessing the methylation of O [6]-methylguanine-DNA methyltransferase (MGMT) while simultaneously detecting the isocitrate dehydrogenase (IDH) mutation status in glioma patients, and another method has been developed for concomitantly detecting CpG methylation and chromatin accessibility [6, 103].

Transposable elements (TEs) are potential etiopathogenic factors in cancer, and DNA methylation is a process that regulates TE activity. Short read mapping has been unable to uncover high-copy-number TEs. However, as nanopore technologies have emerged, the development of methylation maps and telomere-to-telomere chromosome assembly at base-pair resolution has also been useful for identifying TE insertions in the genome in different organs (heart, liver, hippocampus) or tumors [100].

A technique called nanopore sequencing of Nucleosome Occupancy and Methylome (nanoNOMe) has been developed in 2020, aiming to simultaneously analyze chromatin accessibility on long DNA sequences and CpG methylation. This method was useful for evaluations of allele-specific epigenetic states within the genome [104].

Software tools for interpreting DNA methylation data

High-throughput sequencing, including nanopore sequencing, generates large amounts of new DNA methylation data, which can assist in the discovery of new biomarkers for diagnosis in different diseases, but there is an increased need for means of interpreting these enormous sets of data [8]. Specifically, nanopore sequencers, which have not been able to replace previous sequencing techniques yet due to their initial high error rates (5–15%) [84], are now able to generate big datasets in a short time through computational systems that have basecalling softwares incorporated [93].

Through nanopore technology, the sequenced nucleotide motifs can provide corresponding signal data, which is translated into long-read sequences through machine-learning methods. The machine-learning algorithms are able to extract the biological information and detect DNA modifications (through identifying the differences in the electric current intensities generated by modified versus unmodified bases) [6], poly(A) tail length and RNA secondary structures [27], being in a constant state of development and optimization [13].

The electric current patterns which result when bases pass through the nanopore are different from one another and they create what are known as 'squiggles'. To identify differences between modified (e.g. methylated DNA nucleotides) and unmodified bases, the squiggles are analyzed and subjected to basecalling and alignment [6]. Basecalling is a process that translates raw ion current signal data generated by the molecules which pass through the nanopore into short nucleotide sequences (initially 6 DNA bases - k-mer) [27]. The cloud-base Metrichor service was one of the first basecallers, which analyzed the files generated by the sequencing software and generated the analysis results [94]. Nanocall was an offline HMM-based alternative to Metrichor [27]. This was the initial HMM-based approach with a 80% accuracy, but more recent basecalling algorithms which show major improvements in read accuracy (98%) have been developed. They are based on a deep learning approach that combines a convolutional neural network (CNN), connectionist temporal classification, and a recurrent neural network (RNN) [1, 95]. An important aspect is that every time the pore chemistry is updated by ONT, the algorithms have to be trained again, but many tools for training the algorithms on their own data have emerged. In the future, genetic and epigenetic information will probably come out of the sequencing directly, without extra processing, and with the development of basecallers which include modified bases, base modifications will become a standard component of DNA sequencing [25].

Detecting DNA methylation through nanopore sequencing should allow detection of methylations in CpG sites, and it is assumed that within a 10-bp region all CpGs share the same methylation status, but the accuracy varies depending on the genomic region. Methylation-calling tools have been developed for different nanopore pore versions (R7, R9, R10 etc.) in order to detect various DNA modifications, such as 5mC, 5hmC, 6 mA and 4mC. Some of the DNA methylation calling tools developed for nanopore sequencing are: Nanopolish (5mC in CpG and GpC sites), Tombo (5mC, 6 mA, 4mC), SignalAlign (5mC - CpG, 5hmC), Guppy (5mC - CpG, 6 mA), NanoMod (5mC - CpG, 6 mA), DeepMod (5mC - CpG, 6 mA), DeepSignal (6 mA), Megalodon (5mC - CpG), mCaller (6 mA), methBERT, METEORE, DeepMP, NP-SMLR (5mC - GpC) [6, 90], NanoMod, MINES, Nanom6A [1], PoreMeth [69], NanoMethViz [26], nanodisco (for bacterial species and microbiomes) [99]. Nanopolish, Megalodon, DeepSignal and Guppy were benchmarked as having a high accuracy for 5mC detection; Nanopolish, Megalodon and Guppy were the fastest, while Nanopolish and Guppy had the lowest memory usage [6, 90]. Furthermore, as the development of new nanopores is still ongoing and it swiftly evolves as we speak, some of the tools aforementioned were outpaced and are not currently used, while other new softwares are emerging. In this review, we have presented both the emblematic inaugural softwares that have been used successfully in the past, but have since been outdated, and some newly emerged ones that have been developed and that are used at present. For example, even though Megalodon and Guppy are no longer supported tools used for analysis, they have been helpful as models for advanced software tools which have been subsequently deployed. Unfortunately, many of these tools still need large training data or further adjustments for detection improvement [28], while they are also able to detect only specific DNA methylation patterns (e.g. only 5mC at CpG, or only 6 mA modifications) [99].

Nanopolish is a popular software for nanopore sequencing, which can detect multiple methylation patterns through pre-trained algorithms, without the need of PCR amplification of the DNA sample [25]. It can differentiate between unmethylated cytosine and 5mC in CpG and GpC, while also being able to fulfill other roles such as measuring poly(A) tail length [1, 27].

In 2019, **DeepSignal** was released for the detection of 5mC modifications in CpG sites in the human genome. Subsequently, the derived version DeepSignal-plant was developed for identifying 5mC methylation patterns in plant genomes [1].

Nanomod is a software developed in 2020 that can detect de novo DNA modifications, achieving better results than other tools, without the need for training data. The downside, however, is that it is not able to predict the specific type of modification [28].

PoreMeth was developed for detecting DNA-methylation alteration in sample pairs, being capable of analyses of CpG islands and sparse CpGs with a very high resolution (>99% of CpGs) [69].

NanoMethViz is a package that handles long-read methylation data, offering extended visualization options at various resolutions for nanopore data generated through sequencing. This software converts the output data obtained through basecalling softwares such as Nanopolish and Megalodon into formats which are compatible with Bioconductor packages, for in-detail DNA methylation analyses [26].

As of August 2024, Oxford Nanopore Technologies have listed MinKNOW as the operating software which integrates basecalling, and Dorado as the official basecaller which is also available on MinKNOW.

MinKNOW integrates three basecalling models: a fast model keeping up with raw data generation from MinION Mk1C, GridION and PromethION, a high accuracy model providing an enhanced raw read accuracy, and a super accurate model which is even more intensive. Through MinKNOW, the user has the ability to access different basecalling models trained to identify base modifications, such as 5mC+5hmC (CG-context and all-context) and 6 mA (all-context) for DNA, and a m6A model for RNA.

Dorado is a data processing toolkit containing basecalling algorithms and post-processing features, which implements a neural networks algorithm allowing raw data transformation into bases of DNA, RNA, or modified bases. It includes models for 5mC+5hmC, 6 mA, 4mC+5mC for DNA, and m6A, pseudouridine models for RNA, and it can be used on Windows, Mac OS X, and on multiple Linux platforms [105].

There is a need for improving the computational efficiency of basecalling, since the trend is for developing larger deep learning-based models for improved accuracy which consume very high amounts of power. These deep learning-based basecallers currently need to be tuned manually through trial and error by computational biologists, and therefore they cannot focus on speed. In February 2024, **RUBICON** was developed as the first framework for creating hardware-optimized basecallers. It includes two machine learning techniques (QABAS and SkipClip), which ultimately ensure basecalling accuracy while reducing resource and storage requirements [106].

Methylation levels can display high levels of spatial correlation, with the majority of cytosines within a specific distance (e.g., 1 Kb in humans) displaying similar methylation values [107]. For many applications (clinical and research), one needs to identify differentially methylated

regions (DMRs), which are regions of the genome that display differential methylation at multiple neighboring cytosines between two conditions. There are a several tools that have been developed to detect DMRs for WGBS, including: methylKit [108], DMRcaller [107], DSS [109], methylSig [110], BiSeq [111], bsseq [112], methylPipe [113], RnBeads [114], BEAT [115] or M3D [116]. These tools can also be applied to DNA methylation data generated by Nanopore sequencing. Nevertheless, while these tools that were developed for WGBS can generate accurate DMR annotation for Nanopore sequencing methylation data, they will not capture the unique information in the long read. For example, with long reads, there is an opportunity to detect whether a decrease in cytosine methylation in a region happens homogeneously (fewer DNA molecules are completely methylated) or heterogeneously (there is a reduction in the percentage of methylated cytosine on each DNA molecule). These types of tools are needed to enhance our understanding of how DNA methylation controls gene transcription.

Other software tools meant to improve the quality of nanopore sequencing have started to emerge and will probably be developed in the future. For example, in April 2024, a tool called NextDenovo was introduced for error correction and assembling long reads, managing to improve the level of genome-assembly accuracy [117].

Conclusions

The current modern techniques which are capable of sequencing the entire human genome have paved the way for new scientific discoveries, as they are a means of providing answers to long-time open questions. The detection of DNA methylation, which is an epigenetic modification responsible for many physiological and pathological processes in the human body, is very important for understanding the mechanisms of gene expression, as well as for adding complexity to traditional points-of-view. For example, aberrant DNA methylations have been associated with genetic instability and development of malignant tumors, and this epigenetic modification might serve as a target for new therapeutic strategies [18]. Methylated genes might serve as cancer biomarkers, and a few such examples already used in clinical practice are hypermethylated septin 9 (SEPT9) in colorectal cancer and abnormal methylation of glutathione S-transferase pi 1 (GSTP1) in prostate cancer [13]. DNA methylation-based in vitro diagnostic assays and liquid biopsy tests are currently in development, as this new trend has emerged for multi-cancer detection [13, 70].

Great progress has been made especially through nanopore sequencing and its multitude of associated bioinformatic softwares that provide accessible means of detecting and studying methylations along the complete genome. Through nanopore sequencing, nucleotides can be detected without the prior need of chemically treatments on the DNA strands, which is a step forward from bisulfite sequencing, and it is able to detect full-length transcripts. However, alike every other sequencing technique, there are still a few obstacles to overcome, such as the need of training the bioinformatic tools with large sets of data prior to the analyses and more tools to take advantage of long reads in analyzing differentially methylated regions.

In conclusion, nanopore sequencing has started to shed light on puzzling genomic and epigenomic matters, and it is highly likely that in the nearest future scientific breakthroughs regarding DNA methylation and its roles will be made, finally answering the unanswered questions on gene expression and malignant transformation.

Abbreviations

DNA	Deoxyribonucleic acid
NGS	Next-generation sequencing
5mC	5-methylCytosine
5hmC	5-hydroxyMethylcytosine
ONT	Oxford Nanopore Technologies
SMRT	Single molecule real-time
PacBio	Pacific Biosciences
A	Adenine
C	Cytosine
G	Guanine
T	Thymine
DNMTs	DNA methyltransferase enzymes
SAM	S-adenosyl methionine
TET	Ten-eleven translocation
AID	Activation-induced cytidine deaminase
mRNA	Messenger ribonucleic acid
APOBEC	Apolipoprotein B messenger ribonucleic acid editing enzyme, catalytic polypeptide
MBD	Methyl-CpG-binding domain
UHRF	Ubiquitin-like containing PHD and RING finger domains
5hmC	5-hydroxymethylcytosine
P	Phosphate
CpG	Cytosine-phosphate-guanine
CGI	CpG island
6mA	N6-methyladenine
4mC	N4-methylcytosine
5fC	5-formylcytosine
5caC	5-carboxylcytosine
ICF syndrome	Immunodeficiency, Centromere instability and Facial anomalies syndrome
ATRX syndrome	Alpha thalassemia X-linked intellectual disability
PofO	Parent-of-origin
DMRs	Differentially methylated regions
pRB	Retinoblastoma protein
cfDNA	Circulating cell-free DNA
miRNA	Micro-ribonucleic acid
PCR	Polymerase chain-reaction
SNP	Single-nucleotide polymorphisms
SMRT	Single-molecule real-time
LRS	Long-read sequencing
ONT	Oxford Nanopore Technologies
MspA	Mycobacterium smegmatis porin A
Si_3N_4	Silicon nitride
SiO_2	Silicon dioxide
Al_2O_3	Aluminum oxide
BN	Boron nitride
HMM	Hidden Markov models
MGMT	O^6-methylguanine-DNA methyltransferase

TE	Transposable element
nanoNOMe	Nanopore sequencing of Nucleosome Occupancy and Methylome
CNN	Convolutional neural network
RNN	Recurrent neural network
SEPT9	Septin 9
GSTP1	Glutathione S-transferase pi 1

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Author contributions
AC summarized the literature data, organized the review, created the figures and wrote the first draft of this article. NRZ helped in writing the manuscript, while also substantially revising the first draft. OB, NRZ and MSC supervised the work and contributed to the design, organization and writing of the manuscript. The authors have all read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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References

1. Xie S, Leung AWS, Zheng Z, et al. Applications and potentials of nanopore sequencing in the (epi)genome and (epi)transcriptome era. Innov (Camb). 2021;2(4):100153. https://doi.org/10.1016/j.xinn.2021.100153.
2. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. Nature. 2001;409(6822):860–921. https://doi.org/10.1038/35057062.
3. International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of the human genome. Nature. 2004;431(7011):931–45. https://doi.org/10.1038/nature03001.
4. Devuyst O. The 1000 Genomes Project: welcome to a New World. Perit Dial Int. 2015;35(7):676–7. https://doi.org/10.3747/pdi.2015.00261.
5. 1000 Genomes Project Consortium, Auton A, Brooks LD, et al. A global reference for human genetic variation. Nature. 2015;526(7571):68–74. https://doi.org/10.1038/nature15393.
6. Liu Y, Rosikiewicz W, Pan Z, et al. DNA methylation-calling tools for Oxford Nanopore sequencing: a survey and human epigenome-wide evaluation. Genome Biol. 2021;22(1):295. https://doi.org/10.1186/s13059-021-02510-z.
7. Akbari V, Garant JM, O'Neill K, et al. Genome-wide detection of imprinted differentially methylated regions using nanopore sequencing. Elife. 2022;11:e77898. https://doi.org/10.7554/eLife.77898.
8. von Känel T, Huber AR. DNA methylation analysis. Swiss Med Wkly. 2013;143:w13799. https://doi.org/10.4414/smw.2013.13799.
9. De Mendoza A, Nguyen TV, Ford E, et al. Large-scale manipulation of promoter DNA methylation reveals context-specific transcriptional responses and stability. Genome Biol. 2022;23(1):163. https://doi.org/10.1186/s13059-022-02728-5.
10. Grant OA, Wang Y, Kumari M, Zabet NR, Schalkwyk L. Characterising sex differences of autosomal DNA methylation in whole blood using the Illumina EPIC array. Clin Epigenet. 2022;14(1):62. https://doi.org/10.1186/s13148-022-01279-7.
11. Grant OA, Kumari M, Schalkwyk L, Zabet NR. Systematic investigation of inter-individual variation of DNA methylation in human whole blood. Published Online January. 2024;31. https://doi.org/10.1101/2024.01.29.577703.
12. Moore L, Thuc L, Guoping F. DNA methylation and its basic function. Neuropsychopharmacology: Official Publication Am Coll Neuropsychopharmacol. 2013;38(1). https://doi.org/10.1038/npp.2012.112.
13. Martisova A, Holcakova J, Izadi N, Sebuyoya R, Hrstka R, Bartosik M. DNA methylation in solid tumors: functions and methods of detection. Int J Mol Sci. 2021;22(8):4247. https://doi.org/10.3390/ijms22084247.
14. Bearzatto A, Conte D, Frattini M, et al. p16(INK4A) hypermethylation detected by fluorescent methylation-specific PCR in plasmas from non-small cell lung cancer. Clin Cancer Res. 2002;8(12):3782–7.
15. Yu J, Ni M, Xu J, et al. Methylation profiling of twenty promoter-CpG islands of genes which may contribute to hepatocellular carcinogenesis. BMC Cancer. 2002;2:29. https://doi.org/10.1186/1471-2407-2-29.
16. Mattei AL, Bailly N, Meissner A. DNA methylation: a historical perspective. Trends Genet. 2022;38(7):676–707. https://doi.org/10.1016/j.tig.2022.03.010.
17. Johnson TB, Coghill RD, RESEARCHES, ON PYRIMIDINES. C111. THE DISCOVERY OF 5-METHYL-CYTOSINE IN TUBERCULINIC ACID, THE NUCLEIC ACID OF THE TUBERCLE BACILLUS1. J Am Chem Soc. 1925;47(11):2838–44. https://doi.org/10.1021/ja01688a030.
18. Meng H, Cao Y, Qin J, et al. DNA methylation, its mediators and genome integrity. Int J Biol Sci. 2015;11(5):604–17. https://doi.org/10.7150/ijbs.11218.
19. Hotchkiss RD, The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. J Biol Chem. 1948;175(1):315–32. https://doi.org/10.1016/S0021-9258(18)57261-6.
20. Taylor SM, Jones PA. Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell. 1979;17(4):771–9. https://doi.org/10.1016/0092-8674(79)90317-9.
21. Waddington CH. The epigenotype. 1942. Int J Epidemiol. 2012;41(1):10–13. https://doi.org/10.1093/ije/dyr184
22. Martienssen RA, Riggs AD, Russo VEA. Epigenetic mechanisms of Gene Regulation. Cold Spring Harbor (N.Y). Cold Spring Harbor laboratory; 1996. https://lib.ugent.be/catalog/rug01:000443185.
23. Frommer M, McDonald LE, Millar DS, et al. A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U S A. 1992;89(5):1827–31. https://doi.org/10.1073/pnas.89.5.1827.
24. Silva C, Machado M, Ferrão J, Sebastião Rodrigues A, Vieira L. Whole human genome 5'-mC methylation analysis using long read nanopore sequencing. Epigenetics. 2022;17(13):1961–75. https://doi.org/10.1080/15592294.2022.2097473.
25. Gouil Q, Keniry A. Latest techniques to study DNA methylation. Essays Biochem. 2019;63(6):639–48. https://doi.org/10.1042/EBC20190027.
26. Su S, Gouil Q, Blewitt ME, Cook D, Hickey PF, Ritchie ME. NanoMethViz: an R/Bioconductor package for visualizing long-read methylation data. PLoS Comput Biol. 2021;17(10):e1009524. https://doi.org/10.1371/journal.pcbi.1009524.
27. Wan YK, Hendra C, Pratanwanich PN, Göke J. Beyond sequencing: machine learning algorithms extract biology hidden in Nanopore signal data. Trends Genet. 2022;38(3):246–57. https://doi.org/10.1016/j.tig.2021.09.001.
28. Liu Q, Georgieva DC, Egli D, Wang K. NanoMod: a computational tool to detect DNA modifications using Nanopore long-read sequencing data. BMC Genomics. 2019;20(Suppl 1):78. https://doi.org/10.1186/s12864-018-5372-8.
29. Eckersley-Maslin MA, Alda-Catalinas C, Reik W. Dynamics of the epigenetic landscape during the maternal-to-zygotic transition. Nat Rev Mol Cell Biol. 2018;19(7):436–50. https://doi.org/10.1038/s41580-018-0008-z.
30. Wei A, Wu H. Mammalian DNA methylome dynamics: mechanisms, functions and new frontiers. Development. 2022;149(24):dev182683. https://doi.org/10.1242/dev.182683.

31. Law PP, Holland ML. DNA methylation at the crossroads of gene and environment interactions. Essays Biochem. 2019;63(6):717–26. https://doi.org/10.1042/EBC20190031.
32. Michaud EJ, van Vugt MJ, Bultman SJ, Sweet HO, Davisson MT, Woychik RP. Differential expression of a new dominant agouti allele (Aiapy) is correlated with methylation state and is influenced by parental lineage. Genes Dev. 1994;8(12):1463–72. https://doi.org/10.1101/gad.8.12.1463.
33. Pal D, Patel M, Boulet F, et al. H4K16ac activates the transcription of transposable elements and contributes to their cis-regulatory function. Nat Struct Mol Biol. 2023;30(7):935–47. https://doi.org/10.1038/s41594-023-01016-5.
34. Weber M, Hellmann I, Stadler MB, et al. Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet. 2007;39(4):457–66. https://doi.org/10.1038/ng1990.
35. D H, Z L, C W, et al. N6-methyladenine-mediated aberrant activation of the lncRNA SOX2OT-GLI1 loop promotes non-small-cell lung cancer stemness. Cell Death Discovery. 2023;9(1). https://doi.org/10.1038/s41420-023-01442-w.
36. Y Z, Z C. The role of m6A modified circ0049271 induced by MNNG in precancerous lesions of gastric cancer. Heliyon. 2024;10(16). https://doi.org/10.1016/j.heliyon.2024.e35654.
37. Yu X, Ren J, Cui Y, Zeng R, Long H, Ma C. DRSN4mCPred: accurately predicting sites of DNA N4-methylcytosine using deep residual shrinkage network for diagnosis and treatment of gastrointestinal cancer in the precision medicine era. Front Med (Lausanne). 2023;10:1187430. https://doi.org/10.3389/fmed.2023.1187430.
38. Storebjerg TM, Strand SH, Høyer S, et al. Dysregulation and prognostic potential of 5-methylcytosine (5mC), 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) levels in prostate cancer. Clin Epigenetics. 2018;10(1):105. https://doi.org/10.1186/s13148-018-0540-x.
39. Liao J, Karnik R, Gu H, et al. Targeted disruption of DNMT1, DNMT3A and DNMT3B in human embryonic stem cells. Nat Genet. 2015;47(5):469–78. https://doi.org/10.1038/ng.3258.
40. Bestor T, Laudano A, Mattaliano R, Ingram V. Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. J Mol Biol. 1988;203(4):971–83. https://doi.org/10.1016/0022-2836(88)90122-2.
41. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell. 1999;99(3):247–57. https://doi.org/10.1016/s0092-8674(00)81656-6.
42. Barau J, Teissandier A, Zamudio N, et al. The DNA methyltransferase DNMT3C protects male germ cells from transposon activity. Science. 2016;354(6314):909–12. https://doi.org/10.1126/science.aah5143.
43. Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. Nature. 2010;466(7310):1129–33. https://doi.org/10.1038/nature09303.
44. Hata K, Okano M, Lei H, Li E. Dnmt3L cooperates with the Dnmt3 family of de novo DNA methyltransferases to establish maternal imprints in mice. Development. 2002;129(8):1983–93. https://doi.org/10.1242/dev.129.8.1983.
45. Aapola U, Kawasaki K, Scott HS, et al. Isolation and initial characterization of a novel zinc finger gene, DNMT3L, on 21q22.3, related to the cytosine-5-methyltransferase 3 gene family. Genomics. 2000;65(3):293–8. https://doi.org/10.1006/geno.2000.6168.
46. Kovacheva VP, Mellott TJ, Davison JM, et al. Gestational choline deficiency causes global and Igf2 gene DNA hypermethylation by up-regulation of Dnmt1 expression. J Biol Chem. 2007;282(43):31777–88. https://doi.org/10.1074/jbc.M705539200.
47. Issa JPJ, Kantarjian HM, Kirkpatrick P, Azacitidine. Nat Rev Drug Discov. 2005;4(4):275–6. https://doi.org/10.1038/nrd1698.
48. Gore SD, Jones C, Kirkpatrick P, Decitabine. Nat Rev Drug Discov. 2006;5(11):891–2. https://doi.org/10.1038/nrd2180.
49. Gigante S, Gouil Q, Lucattini A, et al. Using long-read sequencing to detect imprinted DNA methylation. Nucleic Acids Res. 2019;47(8):e46. https://doi.org/10.1093/nar/gkz107.
50. Li S, Chen X, Wang J, et al. Somatic mutations drive specific, but reversible, Epigenetic Heterogeneity States in AML. Cancer Discov. 2020;10(12):1934–49. https://doi.org/10.1158/2159-8290.CD-19-0897.
51. Li Q, Chen S, Leung AWS et al. DNA methylation affects pre-mRNA transcriptional initiation and processing in Arabidopsis. Published online April 30, 2021:2021.04.29.441938. https://doi.org/10.1101/2021.04.29.441938
52. Conlin LK, Aref-Eshghi E, McEldrew DA, Luo M, Rajagopalan R. Long-read sequencing for molecular diagnostics in constitutional genetic disorders. Hum Mutat. 2022;43(11):1531–44. https://doi.org/10.1002/humu.24465.
53. Yu B, Qi Y, Li R, Shi Q, Satpathy AT, Chang HY. B cell-specific XIST complex enforces X-inactivation and restrains atypical B cells. Cell. 2021;184(7):1790–e180317. https://doi.org/10.1016/j.cell.2021.02.015.
54. Akbari V, Hanlon VCT, O'Neill K, et al. Parent-of-origin detection and chromosome-scale haplotyping using long-read DNA methylation sequencing and strand-seq. Cell Genom. 2023;3(1):100233. https://doi.org/10.1016/j.xgen.2022.100233.
55. Xie W, Barr CL, Kim A, et al. Base-resolution analyses of sequence and parent-of-origin dependent DNA methylation in the mouse genome. Cell. 2012;148(4):816–31. https://doi.org/10.1016/j.cell.2011.12.035.
56. Fang F, Hodges E, Molaro A, Dean M, Hannon GJ, Smith AD. Genomic landscape of human allele-specific DNA methylation. Proc Natl Acad Sci U S A. 2012;109(19):7332–7. https://doi.org/10.1073/pnas.1201310109.
57. Busato F, Tost J. SNP-Based quantification of allele-specific DNA methylation patterns by Pyrosequencing®. Methods Mol Biol. 2015;1315:291–313. https://doi.org/10.1007/978-1-4939-2715-9_21.
58. Yamada M, Okuno H, Okamoto N, et al. Diagnosis of Prader-Willi syndrome and Angelman syndrome by targeted nanopore long-read sequencing. Eur J Med Genet. 2023;66(2):104690. https://doi.org/10.1016/j.ejmg.2022.104690.
59. Wells JN, Feschotte C. A Field Guide to eukaryotic transposable elements. Annu Rev Genet. 2020;54(1):539–61. https://doi.org/10.1146/annurev-genet-040620-022145.
60. Hayward A, Gilbert C. Transposable elements. Curr Biol. 2022;32(17):R904–9. https://doi.org/10.1016/j.cub.2022.07.044.
61. Bhat A, Ghatage T, Bhan S, et al. Role of transposable elements in Genome Stability: implications for Health and Disease. IJMS. 2022;23(14):7802. https://doi.org/10.3390/ijms23147802.
62. Wang Z, Ying Y, Wang M, et al. Comprehensive identification of onco-exaptation events in bladder cancer cell lines revealed L1PA2-SYT1 as a prognosis-relevant event. iScience. 2023;26(12). https://doi.org/10.1016/j.isci.2023.108482.
63. Jing T, Wei D, Xu X, et al. Transposable elements-mediated recruitment of KDM1A epigenetically silences HNF4A expression to promote hepatocellular carcinoma. Nat Commun. 2024;15(1):5631. https://doi.org/10.1038/s41467-024-49926-2.
64. Singh B, Dopkins N, Fei T, et al. A transposable element atlas of aggressive B-cell non-hodgkin lymphomas defines novel classifications of Burkitt lymphoma independent of EBV status. Hematol Oncol. 2023;41(S2):247–247. https://doi.org/10.1002/hon.3164_160.
65. Lemerle E, Trompouki E. Transposable elements in normal and malignant hematopoiesis. Dis Models Mech. 2023;16(8):dmm050170. https://doi.org/10.1242/dmm.050170.
66. Valdebenito-Maturana B, Rojas-Tapia MI, Carrasco M, Tapia JC. Dysregulated expression of transposable elements in TDP-43M337V human motor neurons that recapitulate amyotrophic lateral sclerosis in Vitro. IJMS. 2022;23(24):16222. https://doi.org/10.3390/ijms232416222.
67. Jang HS, Shah NM, Du AY, et al. Transposable elements drive widespread expression of oncogenes in human cancers. Nat Genet. 2019;51(4):611–7. https://doi.org/10.1038/s41588-019-0373-3.
68. Paulson H. Repeat expansion diseases. Handb Clin Neurol. 2018;147:105–23. https://doi.org/10.1016/B978-0-444-63233-3.00009-9.
69. Magi A, Mattei G, Mingrino A, et al. High-resolution Nanopore methylome-maps reveal random hyper-methylation at CpG-poor regions as driver of chemoresistance in leukemias. Commun Biol. 2023;6(1):382. https://doi.org/10.1038/s42003-023-04756-8.
70. Locke WJ, Guanzon D, Ma C, et al. DNA methylation Cancer biomarkers: translation to the clinic. Front Genet. 2019;10:1150. https://doi.org/10.3389/fgene.2019.01150.
71. Sakamoto Y, Zaha S, Nagasawa S, et al. Long-read whole-genome methylation patterning using enzymatic base conversion and nanopore sequencing. Nucleic Acids Res. 2021;49(14):e81. https://doi.org/10.1093/nar/gkab397.
72. Sterlacci W, Tzankov A, Veits L, et al. A comprehensive analysis of p16 expression, gene status, and promoter hypermethylation in surgically resected non-small cell lung carcinomas. J Thorac Oncol. 2011;6(10):1649–57. https://doi.org/10.1097/JTO.0b013e3182295745.
73. Esteller M, Corn PG, Baylin SB, Herman JG. A gene hypermethylation profile of human cancer. Cancer Res. 2001;61(8):3225–9.
74. Catteau A, Harris WH, Xu CF, Solomon E. Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease

characteristics. Oncogene. 1999;18(11):1957–65. https://doi.org/10.1038/sj.onc.1202509.
75. Poojary M, Jishnu PV, Kabekkodu SP. Prognostic Value of Melanoma-Associated Antigen-A (MAGE-A) Gene expression in various human cancers: a systematic review and Meta-analysis of 7428 patients and 44 studies. Mol Diagn Ther. 2020;24(5):537–55. https://doi.org/10.1007/s40291-020-00476-5.
76. Colemon A, Harris TM, Ramanathan S. DNA hypomethylation drives changes in MAGE-A gene expression resulting in alteration of proliferative status of cells. Genes Environ. 2020;42:24. https://doi.org/10.1186/s41021-020-00162-2.
77. Hoang NM, Rui L. DNA methyltransferases in hematological malignancies. J Genet Genomics. 2020;47(7):361–72. https://doi.org/10.1016/j.jgg.2020.04.006.
78. Bhogal B, Weir BA, Crescenzo R, et al. The methyltransferase domain of DNMT1 is an essential domain in acute myeloid leukemia independent of DNMT3A mutation. Commun Biol. 2022;5(1):1174. https://doi.org/10.1038/s42003-022-04139-5.
79. Cimmino L, Dolgalev I, Wang Y, et al. Restoration of TET2 function blocks aberrant Self-Renewal and Leukemia Progression. Cell. 2017;170(6):1079–e109520. https://doi.org/10.1016/j.cell.2017.07.032.
80. Shrestha R, Sakata-Yanagimoto M, Maie K, et al. Molecular pathogenesis of progression to myeloid leukemia from TET-insufficient status. Blood Adv. 2020;4(5):845–54. https://doi.org/10.1182/bloodadvances.2019001324.
81. Katsman E, Orlanski S, Martignano F, et al. Detecting cell-of-origin and cancer-specific methylation features of cell-free DNA from Nanopore sequencing. Genome Biol. 2022;23(1):158. https://doi.org/10.1186/s13059-022-02710-1.
82. Stackpole ML, Zeng W, Li S, et al. Cost-effective methylome sequencing of cell-free DNA for accurately detecting and locating cancer. Nat Commun. 2022;13(1):5566. https://doi.org/10.1038/s41467-022-32995-6.
83. Fuks F, Hurd PJ, Deplus R, Kouzarides T. The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. Nucleic Acids Res. 2003;31(9):2305–12. https://doi.org/10.1093/nar/gkg332.
84. Lin B, Hui J, Mao H. Nanopore Technology and its applications in gene sequencing. Biosens (Basel). 2021;11(7):214. https://doi.org/10.3390/bios11070214.
85. Fuks F, Hurd PJ, Deplus R, Kouzarides T. The DNA methyltransferases associate with HP1 and the SUV39H1 histone methyltransferase. Nucleic Acids Res. 2003;31(9):2305–12.
86. Weaver ICG, Cervoni N, Champagne FA, et al. Epigenetic programming by maternal behavior. Nat Neurosci. 2004;7(8):847–54. https://doi.org/10.1038/nn1276.
87. von Kanel T, Gerber D, Schaller A, et al. Quantitative 1-step DNA methylation analysis with native genomic DNA as template. Clin Chem. 2010;56(7):1098–106. https://doi.org/10.1373/clinchem.2009.142828.
88. Delaneau O, Howie B, Cox AJ, Zagury JF, Marchini J. Haplotype estimation using sequencing reads. Am J Hum Genet. 2013;93(4):687–96. https://doi.org/10.1016/j.ajhg.2013.09.002.
89. Vaisvila R, Ponnaluri VKC, Sun Z, et al. Enzymatic methyl sequencing detects DNA methylation at single-base resolution from picograms of DNA. Genome Res. 2021;31(7):1280–9. https://doi.org/10.1101/gr.266551.120.
90. Wang Y, Zhao Y, Bollas A, Wang Y, Au KF. Nanopore sequencing technology, bioinformatics and applications. Nat Biotechnol. 2021;39(11):1348–65. https://doi.org/10.1038/s41587-021-01108-x.
91. Feng Y, Zhang Y, Ying C, Wang D, Du C. Nanopore-based fourth-generation DNA sequencing technology. Genomics Proteom Bioinf. 2015;13(1):4–16. https://doi.org/10.1016/j.gpb.2015.01.009.
92. Sigurpalsdottir BD, Stefansson OA, Holley G, et al. A comparison of methods for detecting DNA methylation from long-read sequencing of human genomes. Genome Biol. 2024;25(1):69. https://doi.org/10.1186/s13059-024-03207-9.
93. Ahmed YW, Alemu BA, Bekele SA, et al. Epigenetic tumor heterogeneity in the era of single-cell profiling with nanopore sequencing. Clin Epigenetics. 2022;14(1):107. https://doi.org/10.1186/s13148-022-01323-6.
94. Hengyun L, Francesca G, Zemin N. Oxford Nanopore MinION sequencing and Genome Assembly. Genom Proteom Bioinform. 2016;14(5). https://doi.org/10.1016/j.gpb.2016.05.004.
95. Kono N, Arakawa K. Nanopore sequencing: review of potential applications in functional genomics. Dev Growth Differ. 2019;61(5):316–26. https://doi.org/10.1111/dgd.12608.
96. Logsdon GA, Vollger MR, Eichler EE. Long-read human genome sequencing and its applications. Nat Rev Genet. 2020;21(10):597–614. https://doi.org/10.1038/s41576-020-0236-x.
97. Nanopore sequencing accuracy | Oxford Nanopore Technologies. Oxford Nanopore Technologies. Accessed October 19. 2024. https://nanoporetech.com/platform/accuracy
98. Jain M, Koren S, Miga KH, et al. Nanopore sequencing and assembly of a human genome with ultra-long reads. Nat Biotechnol. 2018;36(4):338–45. https://doi.org/10.1038/nbt.4060.
99. Tourancheau A, Mead EA, Zhang XS, Fang G. Discovering multiple types of DNA methylation from bacteria and microbiome using nanopore sequencing. Nat Methods. 2021;18(5):491–8. https://doi.org/10.1038/s41592-021-01109-3.
100. Ewing AD, Smits N, Sanchez-Luque FJ, et al. Nanopore sequencing enables comprehensive transposable element epigenomic profiling. Mol Cell. 2020;80(5):915–e9285. https://doi.org/10.1016/j.molcel.2020.10.024.
101. Hennion M, Arbona JM, Cruaud C, et al. Mapping DNA replication with nanopore sequencing. Published Online September. 2018;26:426858. https://doi.org/10.1101/426858.
102. Shah K, Cao W, Ellison CE. Adenine methylation in Drosophila is Associated with the tissue-specific expression of Developmental and Regulatory genes. G3 (Bethesda). 2019;9(6):1893–900. https://doi.org/10.1534/g3.119.400023.
103. Kreibich E, Kleinendorst R, Barzaghi G, Kaspar S, Krebs AR. Single-molecule footprinting identifies context-dependent regulation of enhancers by DNA methylation. Mol Cell. 2023;83(5):787–e8029. https://doi.org/10.1016/j.molcel.2023.01.017.
104. Lee I, Razaghi R, Gilpatrick T, et al. Simultaneous profiling of chromatin accessibility and methylation on human cell lines with nanopore sequencing. Nat Methods. 2020;17(12):1191–9. https://doi.org/10.1038/s41592-020-01000-7.
105. Oxford Nanopore Technologies: Data analysis. Oxford Nanopore Technologies. August 22. 2024. Accessed September 10, 2024. https://nanoporetech.com/document/data-analysis
106. Singh G, Alser M, Denolf K, et al. RUBICON: a framework for designing efficient deep learning-based genomic basecallers. Genome Biol. 2024;25(1):49. https://doi.org/10.1186/s13059-024-03181-2.
107. Catoni M, Tsang JM, Greco AP, Zabet NR. DMRcaller: a versatile R/Bioconductor package for detection and visualization of differentially methylated regions in CpG and non-CpG contexts. Nucleic Acids Res Published Online July. 2018;9. https://doi.org/10.1093/nar/gky602.
108. Akalin A, Kormaksson M, Li S, et al. methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. Genome Biol. 2012;13(10):R87. https://doi.org/10.1186/gb-2012-13-10-r87.
109. Feng H, Conneely KN, Wu H. A bayesian hierarchical model to detect differentially methylated loci from single nucleotide resolution sequencing data. Nucleic Acids Res. 2014;42(8):e69–69. https://doi.org/10.1093/nar/gku154.
110. Park Y, Figueroa ME, Rozek LS, Sartor MA. MethylSig: a whole genome DNA methylation analysis pipeline. Bioinformatics. 2014;30(17):2414–22. https://doi.org/10.1093/bioinformatics/btu339.
111. Hebestreit K, Dugas M, Klein HU. Detection of significantly differentially methylated regions in targeted bisulfite sequencing data. Bioinformatics. 2013;29(13):1647–53. https://doi.org/10.1093/bioinformatics/btt263.
112. Hansen KD, Langmead B, Irizarry RA. BSmooth: from whole genome bisulfite sequencing reads to differentially methylated regions. Genome Biol. 2012;13(10):R83. https://doi.org/10.1186/gb-2012-13-10-r83.
113. Kishore K, De Pretis S, Lister R, et al. methylPipe and compEpiTools: a suite of R packages for the integrative analysis of epigenomics data. BMC Bioinformatics. 2015;16(1):313. https://doi.org/10.1186/s12859-015-0742-6.
114. Assenov Y, Müller F, Lutsik P, Walter J, Lengauer T, Bock C. Comprehensive analysis of DNA methylation data with RnBeads. Nat Methods. 2014;11(11):1138–40. https://doi.org/10.1038/nmeth.3115.
115. Akman K, Haaf T, Gravina S, Vijg J, Tresch A. Genome-wide quantitative analysis of DNA methylation from bisulfite sequencing data. Bioinformatics. 2014;30(13):1933–4. https://doi.org/10.1093/bioinformatics/btu142.
116. Mayo TR, Schweikert G, Sanguinetti G. M3D: a kernel-based test for spatially correlated changes in methylation profiles. Bioinformatics. 2015;31(6):809–16. https://doi.org/10.1093/bioinformatics/btu749.
117. Hu J, Wang Z, Sun Z, et al. NextDenovo: an efficient error correction and accurate assembly tool for noisy long reads. Genome Biol. 2024;25(1):107. https://doi.org/10.1186/s13059-024-03252-4.

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