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Minireview

Challenges in promoter methylation analysis in the new era of translational oncology: a focus on liquid biopsy

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ABSTRACT

Toward the discovery of novel reliable biomarkers, epigenetic alterations have been repeatedly proposed for the diagnosis and the development of therapeutic strategies against cancer. Indeed, for promoter methylation to actively become a tumor marker for clinical use, it must be combined with a highly informative technology evaluated in an appropriate biospecimen. Methodological standardization related to epigenetic research is, in fact, one of the most challenging tasks. Moreover, tissue-based biopsy is being complemented and, in some cases, replaced by liquid biopsy. This review will highlight the advancements made for both pre-analytical and analytical implementation for the prospective use of methylation biomarkers in clinical settings, with particular emphasis on liquid biopsy.

1. Introduction

The carcinogenic process evolves through the constant accumulation of genetic and epigenetic alterations that enable it to evade physiological control mechanisms [1], aided by an active crosstalk with the microenvironment that composes the tumor niche. Undeniably, epigenetics is a second layer of information that orchestrates the flow of information coming from the genome and that is able to define the actual identity of each cell type [2]. This fine-tuned mechanism is mainly based on four pillars such as: DNA methylation levels, histone post-translational modifications (HPTM), non-coding RNAs, 3D chromatin organization. Since epigenetic modifications represent coordinated phenomena that modulate transcription [3], it is mandatory to identify aberrations in the epigenetic landscape connected to human diseases. In fact, it would provide novel biomarkers required to better refine clinical decisions [4]. Epigenetic changes are reported to be among the earliest aberrations to arise during tumor mass formation [5]. More importantly, these mechanisms are frequently bidirectional and reversible, making them attractive molecular drivers able to select treatment interventions, to assess response rate, survival, and time to therapy failure, all essential features of emergent precision medicine [6].

Aim of this review is to examine the challenges and avenues in the use of promoter methylation as a cancer biomarker in clinical settings.

1.1. *DNA methylation in tumorigenesis*

All four pillars of epigenetics can be altered during tumorigenesis [7–10] but the most studied one is certainly cytosines methylation within CpG dinucleotides. CpG sites are inhomogeneously positioned in the human genome: while most of the genome consists of few and unmethylated CpGs, there are some areas of high CpG density referred as CpG islands (CGIs), which accounts for about 2% of the genome [11]. These are ~0.2–1 kb regions located near transcription start sites (TSS) within gene promoters [12]. A large body of literature points that DNA methylation patterns are frequently altered in cancer and that this occurs in a bimodal way: DNA hypomethylation events are typical of transposable elements, centromeres and oncogenes, contributing to genomic instability and increasing aneuploidy; focal CpG hypermethylation is distinctive of *cis*-regulatory elements and is associated with silencing of tumor suppressor genes (Fig. 1).

DNA methylation is a reversible modification able to define or adapt to tumor biology [15,16]. Indeed, DNA methylation is certainly the *primum movens* of tumorigenesis, since both global changes to normal DNA methylation patterns and methylation levels of regulatory regions are disrupted during early stages of tumorigenesis. These mechanisms act in concert with somatic driver mutations to hinder apoptotic messages and foster tumorigenesis. Furthermore, changes in DNA methylation also regulate many facets of later stages of cancer pathophysiology, such as responses to treatments and metastatization

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[17]. Thus, despite the amount of research in this field, the inner mechanisms underlying changes in the pattern of 5-methylcytosine within the genome as a cause or consequence of cancer evolution remain to be proved [18].

In this context, promoter methylation analysis has been repeatedly proposed as a biomarker for the diagnosis and the development of cancer therapeutic strategies [19]. DNA methylation is in fact a binary mark that can provide relevant information about gene functions in different cell types. It is stable and robust toward transient perturbations, it is more stable than RNA-based biomarkers and does not require any special handling [20]. Since it mirrors the crosstalk with the microenvironment, DNA methylation can highlight data on the course of the disease, thus acting as a bio-archive [21].

The first promoter methylation-related biomarker to be identified is definitely the O^6-*methylguanine-DNA methyltransferase (MGMT)* DNA repair gene. Approximately 50% of glioblastomas manifest *MGMT* promoter hypermethylation. Several clinical trials have suggested that patients presenting *MGMT* promoter hypermethylation experience significant benefit with alkylating drug regimens, such as Temozolomide [22]. For this reason, assays targeting *MGMT* promoter hypermethylation have become a standard of care in the management of glioma-burdened patients, being a crucial aspect in selecting the best treatment strategy [19]. Due to the amount of scientific data supporting it, this is one of the first assay to be commercialized [23,24], although there is no univocity in the methodology ranging from purely qualitative to quantitative ones.

There are at least four macro-areas of interest for which promoter methylation could be used for [25]:

- Early diagnosis: to identify those subjects affected by a neoplasm and who should have access to treatment.
- Choice of therapy: promoter methylation analysis can act as a predictive marker and highlight those tumors that are amenable to first-line or alternative therapy.
- Response or non-response to therapy: promoter methylation analysis can allow monitoring of response to treatment or possible emergence of resistance.
- Monitoring of minimal residual disease after treatments such as surgery, adjuvant chemotherapy, or radiotherapy, to pinpoint those patients at increased risk of recurrence.

Over time, it has become increasingly evident that aberrant DNA methylation would be of translational interest because, by contributing to the very first stages of tumorigenesis, it could become a promising approach for cancer detection. Screening methods based on mutations detection are affected by low sensitivity due to the limited number of recurrent mutations in early stage carcinomas and the clonality and heterogeneity of tumor tissue. In contrast, DNA methylation is not as molecularly constrained and, thus, the related assays have a greater ability to detect early stage cancers [22]. Moreover, compared to normal cells, cancer cells show tissue-specific DNA methylation patterns that are widespread throughout the tumor mass.

On the same premises, DNA methylation-based markers could highlight the presence of minimal residual disease prior any other clinical evidence of disease, identifying patients at high risk of relapse. However, data on DNA methylation turned to be less sensitive than mutations-related ones in detecting minimal residual disease [23].

While, to date, DNA methylation has failed to fully represent a valid tool for assessing minimal residual disease, accumulating data have indicated that gene-specific methylation may be a useful clinical marker for patient prognosis stratification. One example is *RASSF1A*, whose inactivation by promoter methylation is associated with poor prognosis in patients with different cancer types [24].

In addition, DNA methylation patterns may be predictive of patients' response to chemotherapy and correlated with clinical outcome. On this subject, a multicenter study authored by Visvanathan et al. identified a gene panel of six targets capable of predicting survival of metastatic breast cancer-burdened women [26]. *BRCA1* promoter hypermethylation in ovarian cancer correlated with improved survival and response to PARP inhibitors (i.e., Olaparib). In fact, sporadic tumors harboring *BRCA1* promoter methylation show a phenotype similar to *BRCA1*-mutated familial tumors, a condition called "BRCAness" [27]. Loss of *BRCA1* expression caused by promoter hypermethylation disrupts BRCA1-associated DNA repair and can sensitize tumors to BRCA1-

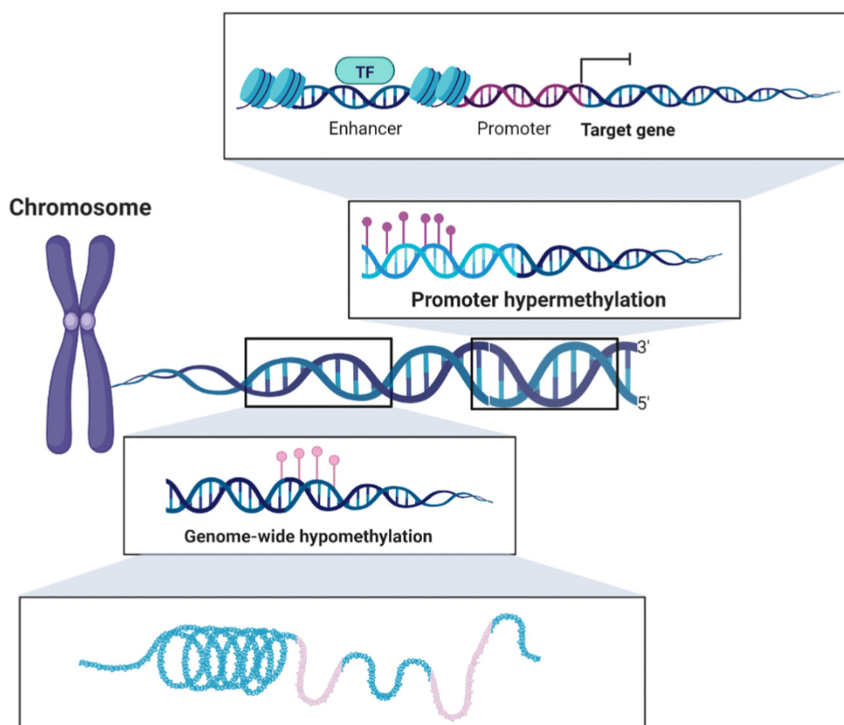

Fig. 1. Schematic representation of the dual DNA methylation pattern across the genome. (Upper panel) focal DNA hypermethylation is typical of CpG islands located in regulatory regions, such as promoters, and associated to gene silencing [13]. (Lower panel) DNA hypomethylation can hinder chromatin compactness leading to genomic instability [14]. Purple marks and DNA stretches represent hypermethylated CpGs; pink marks and DNA stretches represent hypomethylated CpGs. Created with BioRender.com.

directed therapies [28].

Therefore, it seems clear that for promoter methylation to actively become a tumor marker for clinical use, it must be combined with a highly informative technology assessed in an appropriate biospecimen [29]. For primary diagnosis, the assay has to be inexpensive, reliable, minimally-invasive and must have a high specificity and positive predictive value (PPV). When monitoring minimal residual disease or recurrence, the diagnostic test must exhibit the highest sensitivity. Assays evaluating response to therapy and treatment failure should be rapid, allowing immediate management of treatment efficacy. Furthermore, tests should be inexpensive as serial testing is mandatory to uncover the onset of resistance. Finally, diagnostics used for therapeutic intervention should produce a binary outcome tailored to fit the clinical decision-making process around treatment itself [29–31].

Regardless of the goal, the presence of multiple epialleles should be considered [32] since heterogeneous DNA methylation patterns could jeopardize the quantification of promoter methylation. Indeed, whether a particular locus includes n CpG sites, then, there are 2^n possible methylation patterns that are called epialleles [33]. Therefore, DNA methylation at sequential CpG sites can create a signature resembling a cellular barcode.

Hence, a rigorous analytical approach must be performed not only to assess the extent of methylation loading but also to ensure the degree of accuracy required by the clinical output that has to be achieved (see early diagnosis or therapeutic choice) [34].

2. Technological approaches: all that glitters is not gold

The gold standard to assess DNA methylation is based on sodium bisulfite conversion. This procedure allows the conversion of unmethylated cytosines into uracils while methylated ones remain unchanged [35]. The great benefit connected to this technique its undoubted simplicity and the possibility to be used with virtually all molecular biology-based technologies ranging from methylation-specific polymerase chain reaction (MSP), to methylation-sensitive high-resolution melting (MS-HRM), pyrosequencing, methylation-specific droplet digital PCR (MS-ddPCR), microarray, and next generation sequencing (NGS) [36].

MSP relies on primer pairs that are designed to bind unconverted cytosines allowing the amplification of methylated DNA. For a long time, MSP products were analyzed by gel electrophoresis, allowing only qualitative data. In fact, more recent approaches based on the use of TaqMan-based fluorescent probes have made this method quantitative as well. MethyLight is a highly sensitive assay, capable of identifying rare methylated alleles. Primers are designed to analyze regions containing one to three CpGs and, hence, this method is preferred for CGI [37]. This technique is the most widely used to assess targeted promoter methylation.

The methylation-sensitive high-resolution melting (MS-HRM), instead, is based on the comparison of the melting profiles between methylated and unmethylated amplicons [38]. It allows quantification of methylation with a resolution of 5–10% [39]. To avoid primer dimers, amplicons should comprise not more than 100 bp, ensuring high sensitivity but low resolution.

Pyrosequencing is a sequencing-by-synthesis method. It relies on the accumulation of adenosine triphosphate (ATP) related to the incorporation of one nucleotide during a cyclic primer extension step. ATP is, then, used as a substrate for luciferase. Light emission is proportional to the amount of nucleotide incorporated [40,41]. Methylation is inferred by comparing the cytosine-related peak to the thymine-related one for each cytosine in the CpG. This method allows the analysis of regions that are at most 200 bp long, in order to avoid secondary structures and loops that would undermine the analysis [36]. Pyrosequencing is preferred because of its robustness toward CpG poor regions, which may be missed by other techniques.

Droplet digital polymerase chain reaction (ddPCR) is a variant of emulsion-based PCR with ultra-sensitive detection, able to partitioning samples into millions of target-enriched picoliter droplets. Output is based on the average fluorescence intensity of the probe-containing droplets after parallel amplification [42]. ddPCR has recently been optimized to assess promoter hyper-methylation in a quantitative manner by measuring the percentage of the methylated locus within a sample [43].

Targeted CpG sites could be identified with probes on a microarray. They exert a similar approach as single-nucleotide polymorphism microarrays: the probes are directed to the C/T transition created by bisulfite conversion of unmethylated cytosines [44]. The Illumina Infinium MethylationEPIC array is certainly the most widely used microarray for DNA methylation-based investigations. Methylation levels are analyzed after comparing fluorescence intensities of probes targeting the unmethylated and methylated CpGs [45].

Regarding NGS-based techniques, targeted panels could be designed to amplify the region of interest and assess methylation levels. Furthermore, the entire methylome can be analyzed by whole genome bisulfite sequencing (WGBS) or reduced representation bisulfite sequencing (RRBS). In the latter, the DNA is subjected to a digestion step with a methylation-insensitive restriction enzyme and, then, size-selected prior to bisulfite conversion [46]. These techniques are excellent for novel targets discovery, but less convenient for population screening, where a targeted approach is more advisable.

The most important drawbacks of the bisulfite conversion method are associated to the degradation of the material during the harsh temperature conditions that characterize conversion and, in general, the loss of genome complexity due to the imbalance between cytosines and thymines within the converted DNA [35]. Moreover, bisulfite-converted DNA must be processed shortly after bisulfite treatment since long-term storage fosters sample degradation, lowering the sensitivity of DNA methylation-based assays [47]. Lastly, a poor bisulfite conversion rate is usually associated with false positive results.

To overcome these issues, alternative technological approaches have been proposed, such as the one based on methylation-sensitive restriction enzymes (MSRE).

After MSRE cutting, samples are amplified by qPCR to assess the percentage of DNA methylation: the lower the Ct (concentration threshold) the higher the methylation at the cut site within the amplicon [48]. In addition, methylation-dependent enzymes exist. An example is represented by *GlaI* that selectively cuts only methylated DNA, which could be discriminated in end-specific PCR (ES-PCR), designed to amplify only DNA with ends cut by *GlaI* [49].

The use of both methylation-related restriction enzymes has been implemented in the so-called Digital Restriction Enzyme Analysis of Methylation (DREAM). It estimates DNA methylation levels within specific genomic loci contained in the recognition sequence of two restriction enzymes, *SmaI* and *XmaI* [50]. *SmaI* does not perform the cut whether the central CpG site is methylated, whereas *XmaI* cuts both methylated and unmethylated CpG sites. Thus, it is possible to locate the methylated sites since they are the ones not processed by *SmaI* [51]. Restriction enzymes are widely used in clinical diagnostics but this comes with some flaws: lack of robustness, automation, and their semi-quantitative nature.

A different approach relies on affinity capture techniques, which can enrich methylated DNA. It is based on antibody immunoprecipitation using a monoclonal antibody specific for 5-methylcytosine and subsequent investigation by PCR-based methods, microarrays or NGS. The input DNA could be processed in several ways, such as sonication or enzymatic digestion. Methylation-enriched samples are than captured and purified by magnetic beads [52]. Methylated DNA immunoprecipitation (meDIP) is a versatile and unbiased approach that unfortunately needs a large amount of starting material and comes with great costs, which are not always applicable for diagnostic purposes [53].

A summary of the technological approaches used in promoter methylation analysis are depicted in Fig. 2. Notwithstanding the efforts,

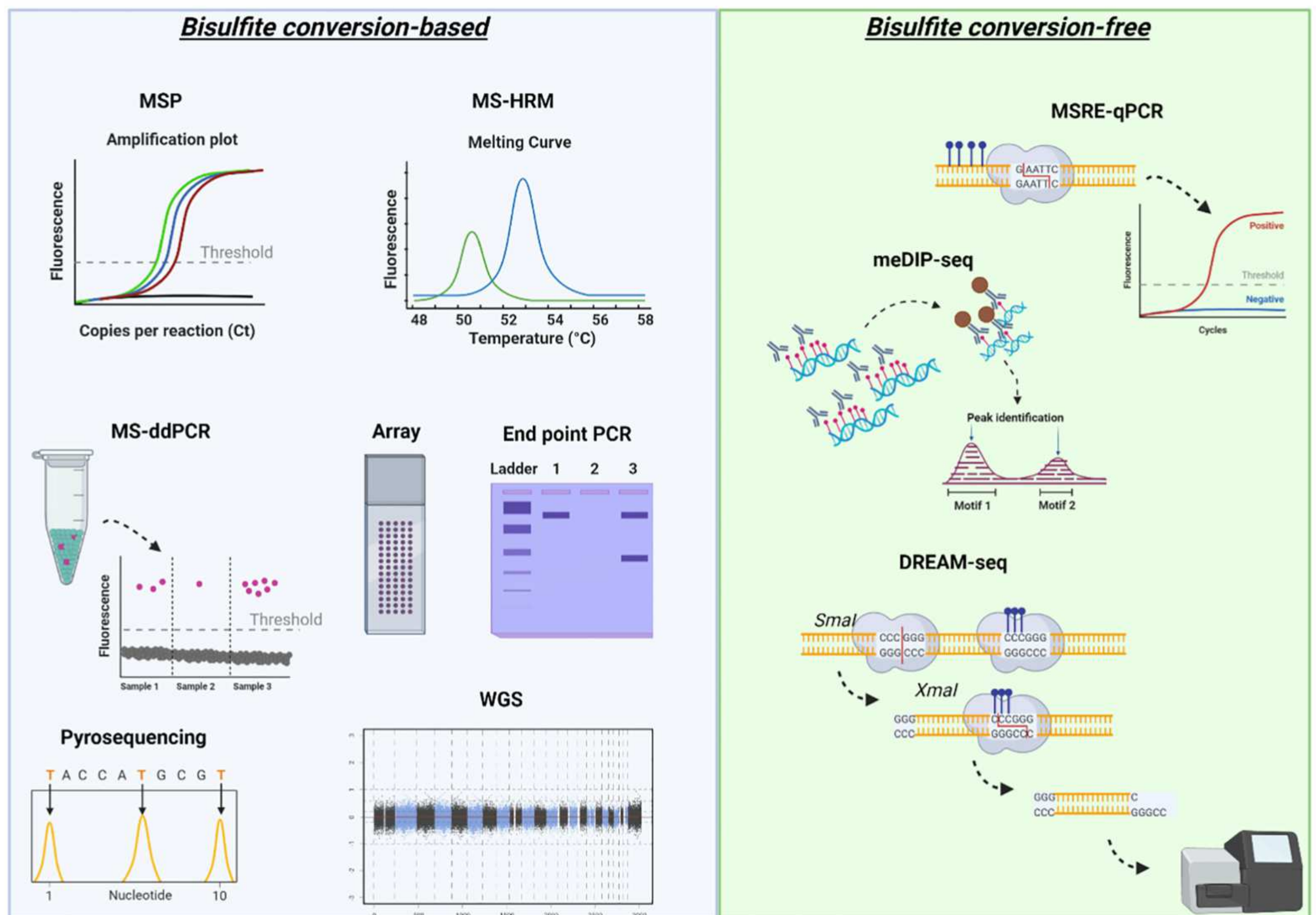

Fig. 2. Methodological approaches to assess promoter methylation in cancer specimens. Technologies meant to assess DNA methylation could be separated into two subcategories: those based on bisulfite conversion of methylated DNA (left panel) and those not involving bisulfite salts (right panel). Abbreviations: DREAM-seq: Digital Restriction Enzyme Analysis of Methylation followed by sequencing; HRM: high resolution melting; meDIP-seq: methylated DNA immunoprecipitation followed by sequencing; MS-ddPCR: methylation specific droplet digital PCR; MSP: methylation specific PCR; MSRE-qPCR: methylation sensitive restriction enzyme digestion followed by PCR; WGS: whole-genome bisulfite sequencing. Created with BioRender.com.

bisulfite conversion still remains the most widely used method to assess DNA methylation [54].

The aforementioned technologies are all useful for the investigation of promoter methylation and their advantages and disadvantages are summarized in Table 2. Overall, targeted technologies are found to be less expensive than global approaches and requires a lesser amount of input material. Moreover, a major drawback of quantitative PCR-based and enzymatic digestion-based approaches is that they can only provide qualitative or semi-quantitative results, which are not always amenable from a clinical perspective. Next-generation based DNA methylation approaches, instead, produce quantitative and single-base resolution data. Despite, DNA methylation profiling platforms differ significantly in their sample quantity and quality requirements. Enzyme-based methods require high quantity, purity and integrity of sample and are useful to assess low-CpG-density regions. DNA purity and integrity are not stringent for the use of antibody-based techniques. These are preferred to analyze CpG-rich regions due to the antibody binding property that allow a low base pair resolution [30,55]. Bisulfite-based profiling methods tends to be sensitive, accurate, reproducible and are capable of single CpG resolution throughout the genome [56]. Notwithstanding, incomplete bisulfite conversion induced by non-proofreading polymerase and re-annealing in PCR amplification may introduce sequencing bias [57].

Therefore, the analytical approach of choice strongly depends on the area of application (see the macro-areas of interest in Section 1.1) not to mention the importance of the sample quality and quantity. Moreover, it should provide an easily interpretable result. Contextually, an important part associated with the use of global approaches to study promoter methylation (i.e., meDIP-seq or WGS) is bioinformatics analysis. Albeit the use of NGS has significantly facilitated the discovery of methylation-based biomarkers, the lack of a uniform pipeline for computational analysis still besets this technology. Whether based on antibody-based enrichment or bisulfite conversion, the analysis procedure should include quality control, alignment, DNA methylation site/peak calling, DNA methylation quantification, and differential methylation site/region (DMS/DMR) identification [31]. Bioinformatic pipelines should also correct data for background noise and tumor heterogeneity.

For all these reasons, methylation-based pipelines require high computational power, memory and storage space and, importantly, expert bioinformatician support to comprehensively analyze and interpret the vast amount of data that is generated [30]. This implies that not all healthcare facilities can meet these needs. Therefore, global methylation analysis such as meDIP-seq, RRBS and WGS are mainly used for biomarker discovery rather than patients' clinical assessment.

3. The tale of the two biopsies: should we go with the flow?

In addition to the technology to be used, the starting material also

plays a critical role in the success of promoter methylation analysis. Traditional approaches include the use of fresh tissue biopsies or formalin-fixed paraffin-embedded (FFPE) samples, which certainly provide a good amount of material to be investigated [58]. FFPE tissues represent an important source of samples in clinical research, chiefly for retrospective studies; therefore, it is mandatory to rely on high-resolution genomic techniques. To date, diverse biomarkers have been assessed and actively used to stratify patients toward cancer aggressiveness or to predict response to treatments [59–65].

The tremendous leaps forward made in the processing and purification of tumor specimens now allow researchers to gather enough material for genome-scale DNA methylation analysis. In fact, DNA restoration protocols are able to recapitulate data from frozen tissues when analyzed with the EPIC array [66].

Despite the advantage, the low quantity and quality of nucleic acids are still the main concerns related to the harsh chemical conditions during the extraction [67]. These limitations seriously affect its use in high-throughput techniques. Although there are continuous implementations of extraction methods, NGS procedures for FFPE samples are still challenging due to nucleic acid fragmentation and artifacts generation during long-term storage [68].

We must keep in mind, however, that tissue biopsy is not always an option as, for example, in the case of limited tissue availability or challenging tumor sites. Anyhow, it is an invasive technique that can certainly not be used to monitor tumor evolution over time [69]: the tumor mass is a spatially and temporally heterogeneous entity that continuously evolves, mainly in response to treatments that spare resistant subclones [70]. On this topic, liquid biopsy is shaping a novel approach for the management of cancer patients. It is in fact well-known that circulating cell-free tumor DNA (ctDNA), circulating miRNAs, circulating tumor cells (CTCs), and tumor-derived extracellular vesicles are released from primary tumors and metastatic sites into body fluids such as peripheral blood, saliva, urine, feces and cerebrospinal fluid (CSF) [71]. Both genetic and epigenetic information could be evaluated, which might provide important information about tumor biology that could be exploited for monitoring and therapeutic purposes. Indeed, circulating cell-free DNA (cfDNA) is a combination of highly fragmented nucleic acids (185–200 bp in length) originating from different tissues and released from necrotic and apoptotic cells into body fluids [72]. Its concentration fluctuates greatly among individuals, but is commonly very low. The fraction of cfDNA shed by the tumor mass is called circulating tumor DNA (ctDNA). Its half-life ranges from 16 min to 2.5 h, and this feature makes it a good tool for dynamic monitoring of the disease burden [73].

To this aim, clinicians must rely on specific and accurate molecular biology-related techniques, since the selected biomarker lies in a sea of confounders represented primarily by the cfDNA poured into body fluids by every single cell composing human body. ctDNA-based liquid biopsy is certainly suitable for evaluating not only genetic but also epigenetic biomarkers, since DNA methylation signatures are highly consistent between ctDNA and genomic DNA derived from the primary tumor [74]. Moreover, changes in DNA methylation signatures in plasma are considered early events during tumorigenesis. A great amount of knowledge has been recently gained about altered promoter methylation patterns in human cancers and tumor-specific promoter methylation changes have been identified and documented in breast cancer, lung cancer, colorectal cancer, head and neck squamous cell carcinoma and hepatocellular carcinoma [74,75].

A substantial slice of translational research has highlighted the prospective role of blood-based DNA methylation patterns in developing assays for early diagnosis, patients stratification and therapeutic monitoring, focusing primarily on quantitative Methylation-Specific PCR (qMSP) [76] and the innovative MS-ddPCR [43,77].

Of the many potential clinical utilities of liquid biopsy, early detection is arguably the most intriguing and ambitious, as very large case–control cohorts are needed to establish the sensitivity and specificity required to accurately identify patients with early-stage disease [78]. In contrast to the highly individualized and heterogeneous nature of the gene mutations, tumor cells of the same type tend to exhibit similar DNA methylation changes, making more feasible the development of blood-based cancer screening campaign. Several studies have reported the detection of putative actionable methylation-based alterations in patients with early-stage disease. Several methylated genes have been proposed as tumor biomarkers for breast cancer detection. Salta et al. demonstrated that aberrant methylation in *FOXA1*, *RASSF1A*, *SCGB3A1*, and *APC* genes differentiated between normal and breast cancer samples with an accuracy of 95.55% [79]. Lung cancer is still associated to a high mortality rate due to later stages diagnosis. Weiss et al. demonstrated that measurement of *SHOX2* and *PTGER4* methylation in plasma DNA allowed detection of lung cancer and differentiation of nonmalignant diseases [60]. A study of Kneip et al. demonstrated that *SHOX2* methylation is able to assess small cell lung cancer with 80% sensitivity and squamous cell carcinoma with 63%, while it fails to diagnose adenocarcinomas since the sensitivity is only 39% [80]. Moreover, Liang et al. developed a methylation based NGS profile able to differentiate lung cancers from benign pulmonary nodules with 75% sensitivity [81]. Other DNA methylation-based candidate biomarker genes for the early detection include *VIM*, *SEPT9* and *SDC2* for colorectal cancer and *GSTP1* for prostate cancer [82] A few large-scale prospective clinical trials are ongoing for multi-cancer early detection by bisulfite sequencing: CCGA (Circulating Cell-free Genome Atlas, NCT02889978), STRIVE (NCT03085888), SUMMIT (NCT03934866) and PATHFINDER (NCT04241796) [83,84]. They reported the lowest sensitivity for stage I cancer detection (18%) while the highest for stage IV cancers (93%) [85]. This is not surprising since patients with early-stage disease may harbor less than one cancer-related molecule per milliliter of biofluid, which is beyond the limit of detection of conventional NGS [78].

Despite all these advantages, to date, only a small number of DNA methylation-based biomarkers (about 14) have been commercialized and even fewer have been approved by the Food and Drug Administration (FDA) or European Medicines Agency [86] (Table 1).

4. Lessons learned from the past

For the diagnosis of colorectal cancer (CRC) two methylation-based tests exist: the stool-based Cologuard® and the blood-based Epi proColon® 2.0. The former consists of a fecal immunochemical test (FIT) coupled with investigation of *KRAS* exon 2 hotspots and assessment of *BMP3* and *NDRG4* promoter hyper-methylation. Epi proColon® 2.0, instead, detects the presence of ctDNA-based SEPT9 promoter methylation using a MethyLight quantitative PCR assay. Although the test has a sensitivity of 75–81% and a specificity of 96–99%, a positive result should always be confirmed by other invasive procedures (i.e., colonoscopy). Despite recent guidelines do not recommend the sole use of Epi proColon® 2.0 for routine CRC screening, this test could be offered to 50 years old patients who refuse colonoscopy [87,88].

Methylation can also be a key element in the assessment of cancer recurrence and an important example is found in non-muscle-invasive bladder cancer (NMIBC), a subtype of cancer in which post-operative surveillance is mandatory as this tumor is characterized by high recurrence rates and a moderate potential of metastatization [29]. Bladder EpiCheck® (Nucleix) is a urine-based ctDNA assay based on methylation-sensitive restriction enzyme digestion coupled with quantitative PCR. It assesses 15 proprietary promoter-based methylation biomarkers, and results are summarized in a score, named EpiScore, ranging from 0 to 100 and related to the propensity of recurrence. This test is widely used for analysis of urine-extracted ctDNA and has a very strong predictive power for recurrence.

The Epi proLung® assay (Epigenomics AG) is the solely in vitro diagnostic (IVD) DNA methylation test for early detection of lung cancer. It assesses *SHOX2* and *PTGER4* promoter hypermethylation on ctDNA from plasma or bronchoalveolar-lavage fluid samples. It has a

Table 1
Strengths and limitations of approaches for methylation analysis.

Technique		Method	Resolution	Advantages	Drawbacks
Global	*WGS*	Bisulfite sequencing	Single base, quantitative	Good coverage of whole genome, easy sample processing	Susceptible to bias from incomplete bisulfite conversion, complex pipeline of analysis
	RRBS	Selection, bisulfite sequencing	Single base, quantitative	Economical, easy sample processing	Susceptible to bias from incomplete bisulfite conversion, low coverage of whole genome
	DREAM-seq	Enzymatic selection, sequencing	Fragment-based, semi-quantitative	Economical, easy sample processing	Large amount of input cfDNA (hundreds of ng), low sensitivity
	meDIP	Antibody-bases enrichment, sequencing	Fragment-based, qualitative	Good coverage of enriched CpGs	Large amount of input cfDNA (hundreds of ng), expensive, time consuming
Targeted	*Endpoint MSP*	Bisulfite conversion, methylation specific amplification	Fragment-based, qualitative	Economical, easy sample processing	Large amount of input cfDNA (hundreds of ng), lack of robustness, low coverage and sensitivity
	qMSP	Bisulfite conversion, methylation specific amplification	Fragment-based, qualitative	Economical, little amount of input cfDNA (ng), easy sample processing	Low coverage and sensitivity
	MS-HRM	Bisulfite conversion, methylation specific melting curve shifting	Temperature-based, quantitative	Economical, little amount of input cfDNA (ng), easy sample processing	Requires the use of known samples to interpolate the percentage of methylation, lack of robustness
	Pyrosequencing	Bisulfite conversion, sequencing-by-synthesis	Single base, quantitative	Economical, little amount of input cfDNA (ng), robust	Small sample processing throughput, time consuming
	MS-ddPCR	Bisulfite conversion, methylation specific amplification	Fragment-based, quantitative	Little amount of input cfDNA (pg), good coverage of selected CpGs, easy sample processing	Expensive, time consuming
	Infinium MethylationEPIC	Bisulfite, array	Single base, quantitative	Economical, good coverage of selected CpGs	Large amount of input cfDNA (hundreds of ng)
	MSRE-qPCR	Enzymatic selection, amplification	Fragment-based, quantitative	Economical, easy sample processing	Large amount of input cfDNA (hundreds of ng), requires the use of known samples to interpolate the percentage of methylation, low sensitivity

Table 2
Currently available IVD/FDA-approved promoter methylation-based biomarkers.

Cancer type	Methylation biomarker	Methodology	Specimen	Commercial name	Year of approval	Clinical utility
Glioblastoma	*MGMT*	Pyrosequencing	FFPE	therascreen® MGMT Pyro® Kit	2012	Predictive
Colorectal cancer	*BMP3, NDRG4*	Quantitative Allele-specific Real-time PCR	Stool	Cologuard®	2014	Diagnosis
Cancers of unknown primary site	Multiple CpG sites	MethylationEPIC array	FFPE and fresh tissue	EPICUP®	2015	Diagnosis/ predictive
Colorectal cancer	*SEPT9*	Real-time PCR with fluorescent hydrolysis probes	Plasma	Epi proColon®	2016	Diagnosis
Lung cancer	*SHOX2, PTGER4*	Real-time PCR with fluorescent hydrolysis probes	Plasma	Epi proLung®	2017	Diagnosis
Bladder cancer	15 methylation biomarkers	Methylation-sensitive restriction enzymes - Real-time PCR	Urine	Bladder EpiCheck®	2017	Surveillance
Glioblastoma	*MGMT*	Real-time PCR with fluorescent hydrolysis probes and methylation-specific primers	FFPE and fresh tissue	MGMT Methylation Detection Kit	2018	Predictive
Breast cancer	*PITX2*	Real-time PCR with fluorescent hydrolysis probes and methylation-unspecific primers	FFPE and fresh tissue	therascreen® PITX2 RGQ PCR Kit	2018	Predictive
Cervical cancer	*ASTN1, DLX1, ITGA4, RXFP3, SOX17, ZNF671*	Real-time PCR with methylation specific primers	Cervical smear	GynTect®	2019	Diagnosis
Hepatocellular carcinoma	*SEPT9*	Real-time PCR with fluorescent hydrolysis probes	Plasma	HCCBloodTest	2019	Diagnosis

sensitivity of 78% and a specificity of 96% [60]. As the Bladder EpiCheck®, a score (EPLT-Score) is calculated to assess the presence of lung carcinoma.

In contrast to the previous examples, promoter methylation has been faintly associated to treatment response, since studies are scarce and highly contradictory [82].

5. The future is only one step away

Hence, liquid biopsy represents a truly promising and noninvasive test to be performed in addition to or in lieu of standard tissue biopsies (Fig. 3). However, huge efforts are still needed before ctDNA-based analyses can be moved from bench to bedside. To be used as a noninvasive monitoring tool in a clinical setting, technical standardization, validation, and improved cohort enrollment are required.

The major obstacle might be the amount of detectable ctDNA. Healthy subjects possess very low amount of cfDNA (4–10 ng/ml of plasma). In cancer patients, ctDNA accounts for approximately 0.01% to 1% of all cfDNA [89], making arduous the assessment of tumor-specific aberrations and therefore requiring to be coupled to highly sensitive techniques. Moreover, several pre-analytical handling factors influence both the quality and the quantity of ctDNA, i.e., leukocyte lysis that occurs during improper sample collection, the delay between collection and analysis (i.e., causing blood clotting in the collection tubes and the release of genomic DNA into the specimen), sample storage conditions and extraction methods [6,89].

Therefore, accuracy, precision, robustness, sensitivity, and specificity should be considered before the use of a liquid biopsy-based test for promoter methylation in diagnostic settings. For instance, fluorometric assays for quantification are highly precise but lowly accurate, especially whether cfDNA is extracted by procedures that use carrier RNA [90]. As a recommendation, it is best to use commercially available

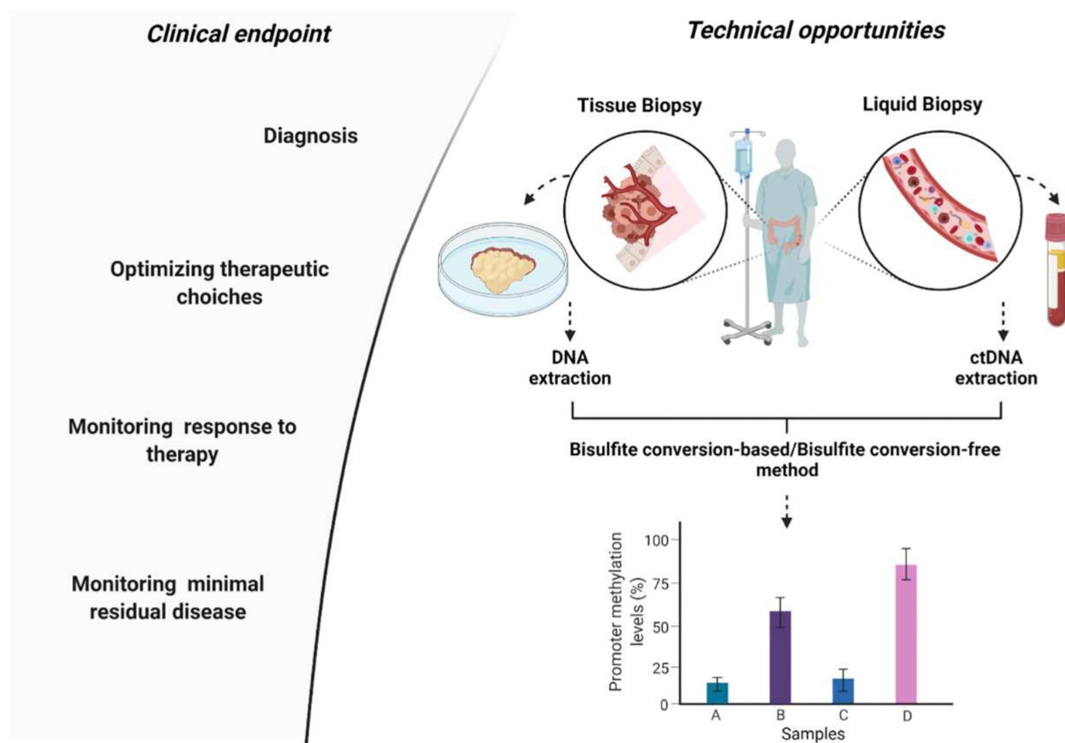

Fig. 3. Management of cancer patients. Precision medicine is based on the assessment of cancer-related biomarkers from two possible specimens (right): tissue biopsies and/or liquid biopsies (mostly plasma based). The ultimate goal is to hit one of the four possible clinical applications (left). Created with BioRender.com.

artificial cfDNA standards to assess sensitivity and specificity when validating a novel approach.

6. Concluding remarks

The application of promoter methylation-based biomarkers is becoming regularly present in clinics. ctDNA-based promoter methylation analysis holds great promise when applied for early diagnosis and to predict and monitor response to specific therapies or the onset of relapse.

Indeed, the introduction of droplet digital PCR has made targeted DNA methylation analysis incredibly feasible, increasing the sensitivity at reasonable cost. However, despite the urge, many improvements are still necessary to develop useful biomarkers [91], especially in the early-stages of cancer.

Early stage tumors are not highly vascularized and that explains the low ctDNA concentration in the blood. Alternative clinical specimens have been proposed to overcome this issue, i.e., the use of urine to diagnose bladder or prostate cancer, sputum for lung cancer, cerebrospinal fluid for glioma, and stool for colorectal cancer [29]. On this topic, the use of NGS-based approaches is mandatory to assess tumor-related signature able to detect stage I cancers and to predict the site of occurrence. Although promising, this strategy produce a multitude of data that requires a highly sophisticated analytical algorithm mostly based on machine learning [92]. While this discipline identifies trends and cancer-specific patterns with ease, it requires massive training data sets and it is excessively time consuming whether it is to be applied to rapid decision-making diagnostics.

Moreover, usable cfDNA is usually limited and vulnerable to contaminations by hemolysis. Furthermore, ctDNA concentration and fragmentation are highly variable due to diverse preanalytical handling conditions, isolation protocols and instruments used, making the development of a worldwide standardized cfDNA analytical procedure imperative.

Therefore, significant effort is still needed to fully realize clinical application of cfDNA methylation markers in the cancer detection and outcome prediction.

Notwithstanding, the continuous technological development of new methods based on promoter methylation-based biomarkers is growing steadily, leading to a fast-paced, innovative, and competitive environment that will result in significant benefits to patient care in the near future.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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