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Review

The role of aberrant DNA methylation in cancer initiation and clinical impacts

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***Abstract*:** Epigenetic alterations, including aberrant DNA methylation, are now recognized as *bone fide* hallmarks of cancer, which can contribute to cancer initiation, progression, therapy responses and therapy resistance. Methylation of gene promoters can have a range of impacts on cancer risk, clinical stratification and therapeutic outcomes. We provide several important examples of genes, which can be silenced or activated by promoter methylation and highlight their clinical implications. These include the mismatch DNA repair genes *MLH1* and *MSH2*, homologous recombination DNA repair genes *BRCA1* and *RAD51C*, the *TERT* oncogene and genes within the *P15/P16/RB1/E2F* tumour suppressor axis. We also discuss how these methylation changes might occur in the first place – whether in the context of the CpG island methylator phenotype or constitutional DNA methylation. The choice of assay used to measure methylation can have a significant impact on interpretation of methylation states, and some examples where this can influence clinical decision-making are presented. Aberrant DNA methylation patterns in circulating tumour DNA (ctDNA) are also showing great promise in the context of non-invasive cancer detection and monitoring using liquid biopsies; however, caution must be taken in interpreting these results in cases where constitutional methylation may be present. Thus, this review aims to provide researchers and clinicians with a comprehensive summary of this broad, but important subject, illustrating the potentials and pitfalls of assessing aberrant DNA methylation in cancer.

Plain language summary

Silencing genes role in initiation of cancer and clinical impacts

Genes can be silenced by molecular tags being placed on them. This is a normal process that controls when and where genes are available to be used. In some cases this silencing can be incorrectly applied to genes involved in preventing cancer, causing cancer initiation and progression. This review discusses the role of one of these tagging processes, DNA methylation and its role in initiation of cancer and implications for treatment.

Keywords: BRCA-related cancers, diagnosis, epigenomics, genomics, genomic testing, homologous recombination deficiency, oncogenic driver mutations, targeted therapy, therapeutic resistance

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Introduction

It is now well accepted that carcinogenesis and cancer proliferation can be driven by both genetic and epigenetic events. Genetic changes directly affect the sequence of the DNA in a cell, whereas epigenetic changes refer to reversible modifications to DNA, histones or RNA and can affect the structure or function of DNA. Thus,

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epigenetic changes do not affect the sequence of the DNA or RNA, but do influence the way that genetic information is read and translated by cells. Some epigenetic modifications to DNA can be passed to daughter cells, and in some cases across generations in organisms.[1] DNA methylation at the fifth carbon of cytosine (5-methylcytosine; 5mC) is a well-studied and highly conserved epigenetic modification that is essential for development in mammals. It can influence gene expression levels, protect cells from repetitive element activity, maintain genome stability during cell division and enable parental gene imprinting. In mammals, cytosine methylation primarily occurs at CpG sites in the DNA sequence.[2,3] Although these CpG sites occur at low frequencies across the mammalian genomes, the majority (60–80%) of them are methylated.[4] Less than 10% of CpG sites are found in CpG dense regions of the genome, known as 'CpG islands', which are often found at transcription start sites of genes. In somatic cells, many CpG islands are unmethylated and this state is associated with the potential for gene expression.[5] However, in the context of cancer, this balance shifts leading to global hypomethylation of the genome coupled with acquired methylation of certain CpG islands.[5] This can lead to silencing of tumour suppressor genes, decreased genomic stability and, in some cases, activation of protooncogenes that can drive carcinogenesis.[5–10] Silencing of tumour suppressor genes *via* promoter hypermethylation are also described as 'epimutations'.[11] Indeed, these epigenetic changes can have similar effects to genetic mutations in the process of cancer development, progression and therapy responses. Specific patterns of CpG methylation have also been associated with different cancer types, and these have been used as biomarkers for cancer detection, prognosis and therapeutic outcomes.[12,13]

Herein, we focus on the impacts of tumour suppressor gene silencing by promoter methylation on cancer initiation and therapeutic responses, with examples provided. We also examine the potential use of unique DNA methylation patterns in cancer for disease risk, diagnosis, monitoring and precision medicine.

Aberrant DNA methylation and carcinogenesis

The first report of tumour suppressor gene silencing contributing to cancer formation was in 1989, describing methylation of the retinoblastoma suppressor gene, *RB1*, in individuals with sporadic unilateral retinoblastoma.[14] Since then, many other tumour suppressor genes have been found to be silenced via promoter methylation in cancers, including *MLH1, BRCA1, RAD51C, APC* and *CDKN2A/p16* (Table 1), and aberrant DNA methylation has been accepted as a feature across multiple cancer types.[15] Like genetic mutations in these genes, epigenetic silencing appears to be enriched in certain cancer types consistent with patterns of genetic mutations, suggesting that both tumour suppressor silencing and mutations can drive carcinogenesis. For example, *MLH1* silencing is found primarily in colorectal[16] and endometrial cancers[17,18], *BRCA1* and *RAD51C* methylation in ovarian and breast cancers, and *APC* methylation in gastrointestinal cancers.[19] Hypermethylation of *CDKN2A/p16* promoter

Table 1. Tumour suppressors known to be silenced by DNA methylation in various cancer types. This non-exhaustive list describes some well-defined tumour suppressor genes silenced (or in the case of THOR, activated) by DNA methylation (either CpG methylation in the promoter or early exons of the gene) across various human cancer types.[19–64]

Gene name	Cellular function	Cancer types	References
APC	Cellular proliferation, migration, DNA repair and chromosomal segregation	Oesophageal, gastric, colorectal, pancreatic, hepatic	Zhu et al.,[19] Barrow et al.[33]
BRCA1	Homologous recombination DNA repair	Ovarian, breast	Cgarn,[23] Cunningham et al.,[24] Kondrashova et al.,[25] Stefansson et al.[27]
RAD51C	Homologous recombination DNA repair	Ovarian, breast, gastric	Cgarn,[23] Cunningham et al.,[24] Nesic et al.[28]
MLH1	Mismatch DNA repair	Colon, gastric, endometrial, non-small cell lung cancer	Zhang et al.,[29] Shen et al.,[30] Post et al.,[31] Idos et al.,[32] Helland et al.[65]
MSH2	Mismatch DNA repair	Colon, gastric, endometrial	Yamamoto and Imai[34]

(Continued)

Table 1. (Continued)

Gene name	Cellular function	Cancer types	References
CDKN2A (p16)	Cell cycle regulation	Oesophageal, gastric, colorectal, pancreatic, lung, bladder, ovarian, breast, melanoma	Wong et al.,[20] Guo et al.,[35] Ye et al.,[36] Tang et al.[37] Sterlacci et al.,[38] Jarmalaite et al.,[39] Bhagat et al.,[40] Spitzwieser et al.,[41] Guo et al.[42]
CDKN2B (p15)	Cell cycle regulation	Leukaemia, liver	Teofili et al.,[43] Ren et al. [44]
INK4-ARF (p14)	Cell cycle regulation	Colorectal, mucoepidermoid carcinoma	Kang et al.,[45] Nikolic et al.[46]
RB1	Cell cycle regulation	Retinoblastoma, glioblastoma, bladder	Benavente and Dyer,[47] Nakamura et al.[48] Malekzadeh et al.[49]
CDH1	Cell adhesion	Breast, thyroid	Zhu et al.,[19] Liu et al.[50]
IGF2	Cell signalling and cell cycle regulation	Wilms' tumours (loss of imprinting, biallelic expression), prostate	Taniguchi et al.,[52] Küffer et al.[53]
STK11/LKB1	Cell polarity, detachment and adhesion, cell structure and energy metabolism	Lung, colorectal, clear cell renal	Zhao and Xu,[54] Koenig et al.,[55] Trojan et al.,[56], Zheng et al.[57]
TIMP3	Cell migration, proliferation and apoptosis	Bladder, kidney, brain, colon, breast, lung	Hoque et al.,[58] Bachman et al.[59]
RASSF1	Cell signalling and cell cycle regulation	Retinblastoma, lung, breast, prostate, glioma, neuroblastoma, kidney	Choy et al.,[60] Hesson et al.,[61] Pfeifer and Dammann[62]
MGMT	DNA repair after alkylating damage	Glioma, retinblastoma	Choy et al.,[60] Yu et al.,[63] Hegi et al.[64]
TERT (THOR)	Cell replication and immortality	Thyroid, skin, bone, ovarian, bladder, lung, brain, prostate, breast, blood, colon	Lee et al.[21]

leads to inactivation of this gene in oesophageal adenocarcinoma.[20] A more comprehensive list of genes and associated cancers is provided in Table 1. Certain proto-oncogenes can also be activated by loss of promoter methylation, for example, *BCL2* in B-cell CLL[6] and *NFATC1* in B-cell CLL,[7] and this phenomenon is discussed in more detail by others.[8–10] However, silencing of the *TERT* oncogene is, in contrast, associated with promoter region hypomethylation.[21,22]

When it comes to tumour suppressor methylation and carcinogenesis, there tends to be a bidirectional interaction between genetic and epigenetic aberrations. Genetic mutations can drive a dysregulated epigenetic landscape, whereas DNA methylation of tumour suppressors can itself be mutagenetic and induce genetic alterations.[66]

Methylation of mismatch DNA repair genes

MLH1 and *MSH2* are important genes in the mismatch DNA repair (MMR) pathway, and their deficiency leads to a specific pattern of genomic hypermutation called microsatellite instability (MSI). Approximately 30% of endometrial cancers,[65] and 15% of colorectal cancers[67,68] exhibit MMR deficiency. Loss of MMR can happen via germline or sporadic mutations in critical MMR genes, and in the case of *MLH1* and *MSH2* this can also occur via promoter methylation and gene silencing (Figure 1).[69–73] In colorectal cancer (CRC), the frequency of *MLH1* promoter methylation is approximately 20% and is particularly associated with right-sided tumours in elderly women.[70] *MLH1* and *MSH2* methylation have also been identified in patients with the inherited cancer syndrome Lynch syndrome.[71–73] The increased MSI resulting from loss of these genes can drive genomic instability and cancer development.[74] MMR is responsible for repair of mismatched DNA bases, short insertions or deletions that can occur during DNA replication.[69] These types of errors are more likely to occur at repetitive regions of the genome, including microsatellites or short tandem repeats.[69] Once MMR is lost, there is an increase of these errors in microsatellites across the genome, leading to mutagenic MSI, which drives cancer development and progression.[69,75]

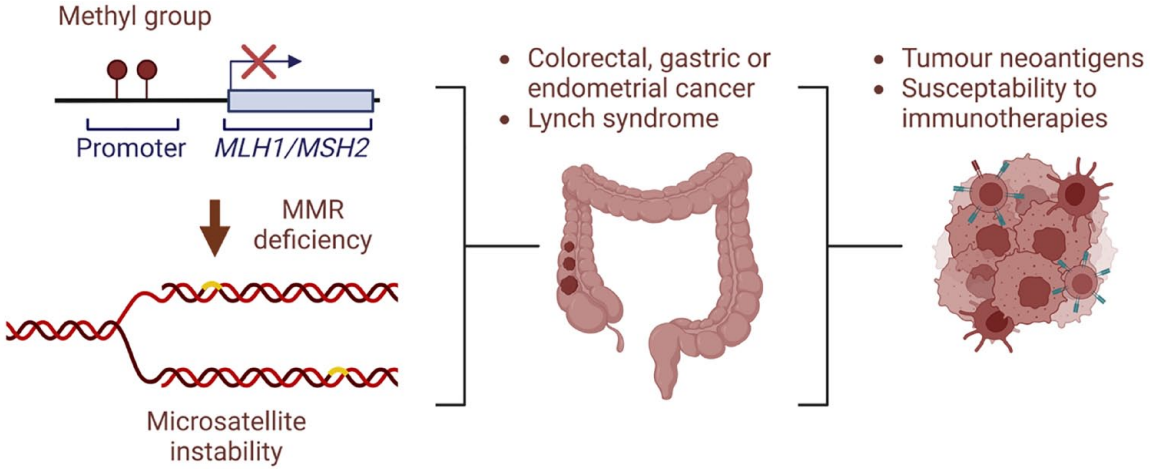

Figure 1. *MLH1/MSH2* gene silencing causes MMR deficiency and drives tumour formation. Loss of MMR via promoter hypermethylation and gene silencing or *MLH1* or *MSH2* causes a form of genomic instability called MSI. This can also be observed for cases with mutations in these genes or other MMR genes *PMS2* and *MLH6*. MSI creates mutations throughout the genome that can drive cancer formation, with colon, gastric and endometrial cancers frequently observed to have MSI. Lynch syndrome due to constitutional epimutations is observed in rare cases.[76] However, MSI can also generate neoantigens that are presented on the tumour cell surface and make these cancers susceptible to immunotherapies.[69,77,78]
MMR, mismatch DNA repair; MSI, microsatellite instability.

MSI resulting from *MLH1* or *MSH2* methylation can, however, have some positive impacts on patient outcomes. High MSI has been demonstrated to have prognostic value in early stage (II) colorectal cancer.[79] It has also been shown to sensitize cells to immune therapy by generating neoantigens that are presented on the surface of cancer cells, distinguishing them from normal cells by the immune system (Figure 1).[77,78] Thus, while methylation of tumour suppressors can have implications for the development of cancer, it can also harbour positive prognostic and therapeutic value in some cases.

Methylation of DNA repair genes involved in homologous recombination

Contrasting implications of tumour suppressor gene methylation can also be observed for the homologous recombination (HR) DNA repair genes *BRCA1* and *RAD51C*. *BRCA1* promoter methylation (me*BRCA1*) and *RAD51C* promoter methylation (me*RAD51C*) are detected in up to 11% and 2% of ovarian cancers, respectively.[23,24] Me*BRCA1* has also been found in approximately 3% of breast cancers,[27] and in the triple-negative breast cancer (TNBC) subtype this can increase up to 22%.[26] Me*RAD51C* may also be found in up to 14% of *BRCA*mutation wildtype TNBC (EMBRACE clinical trial[80,81]). Thus, in these cancer types, me*BRCA1* and me*RAD51C* represent a significant proportion of patients.

The HR pathway is critical for the high-fidelity repair of DNA double strand breaks, which are highly toxic events for cells. When this pathway is inactivated by either mutations of the core genes (e.g. *BRCA1, BRCA2, RAD51C, RAD51D, PALB2*) or silencing of *BRCA1* or *RAD51C*, other more mutagenic pathways of DNA repair compensate.[82] Loss of HR is frequently observed in high-grade serous ovarian cancer and TNBC, usually in the absence of functional *TP53* – often described as the guardian of the genome. The resulting genomic instability can drive not only cancer initiation but also cancer progression. However, as observed for MMR genes, silencing or mutation of these tumour suppressor genes can drive cancer on one hand but is also a positive biomarker for response to platinum chemotherapy and targeted PARP inhibitor (PARPi) therapy on the other hand,[25,28,83,84] and this is covered in more detail later in this review.

In summary, like HR gene mutations, methylation of *BRCA1* and *RAD51C* can not only serve as a driver of cancer but can also be targeted therapeutically, representing a better prognosis for a proportion of these patients if targeted therapy is implemented.

Methylation of P15/P16/RB1/E2F pathway genes

The *P15/P16/RB1/E2F* pathway is one of the most frequently altered pathways in cancer and plays a crucial role in regulating cell cycle progression.[85,86] *CDKN2A* (encoding tumour suppressor p16) and *CDKN2B* (encoding tumour suppressor p15) can negatively regulate CDK4 and CDK6, resulting in hypo-phosphorylation of RB1, which leads to cell cycle arrest. In contrast, hyperphosphorylated RB1 releases activated E2F transcription factor from DNA, thereby initiating DNA replication within S-phase of cell cycle. Thus, P15 and P16 function as tumour suppressors in the late G1 phase of cell cycle, preventing progression to S-phase.[87,88] Mutations in this pathway are frequent across various cancer types.[89] Given the importance of this pathway in suppressing tumour formation, a high degree of regulation has evolved. Thus, it is unsurprising that the *CDKN2A*, *CDKN2B* and *RB1* tumour suppressors can all be silenced by promoter methylation. Methylation of *RB1* has been reported in retinoblastoma, glioblastoma, breast cancer and bladder cancer. Methylation of *CDKN2A* has been reported in oesophageal, gastric, colorectal, pancreatic, lung, bladder, ovarian, breast carcinomas and in melanoma (Table 1). Methylation of *CDKN2B* has been found primarily in leukaemias and lymphomas (Table 1). We focus on a few examples where the prognostic and therapeutic impacts have been assessed.

RB1 hypermethylation has been found in approximately 15% of retinoblastoma tumours and is a key driver of this cancer type.[32] Hypermethylation of *CDKN2A* has been detected in 22–52% of non-small cell lung cancers (NSCLC) and has been shown by several studies to have a prognostic value in NSCLC.[90] For example, in one study, patients with hypermethylated *CDKN2A* had significantly shorter survival (median = 21.7 months) than patients without *CDKN2A* hypermethylation (median = 62.5 months; $p = 0.0001$, log-rank test).[91] In Acute Myeloid Leukaemia (AML), *CDKN2B* methylation has been reported in 49–100% of patients, depending on the population analysed,[92] and has also been found to correlate with poor survival.[93]

CDKN2A or *CDKN2B* methylation can cause increased levels of CDK4/6, which in turn drives hypo-phosphorylation of RB1, suggesting that the CDK4/6 inhibitors may be a useful targeted therapy for *CDKN2A*/*CDKN2B*-methylated cancers.[94,95] As RB1 is downstream of CDK4/6 in this pathway, CDK4/6 inhibitors would not have an effect in cancers with other mechanisms of RB1 inactivation. Demethylating therapies have been recommended as a therapeutic strategy for AML patients with *CDKN2B* methylation.[92] This approach has demonstrated success in a colon cancer cell line, where the DNMT1 inhibitor decitabine could cause demethylation of *CDKN2A*, which appeared to trigger senescence of cancer cells.[96] However, it should be noted that DNMT1 inhibitors like decitabine cause global hypomethylation in cells; thus, it would be difficult to assign their efficacy to demethylation of a single gene or locus.

TERT oncogene promoter methylation

Methylation of the *TERT* promoter region is an interesting exception to the examples provided so far, in that the methylation of this region leads to increased expression the *TERT* oncogene, rather than gene silencing.[21] Telomerase reverse transcriptase, encoded by the *TERT* gene, forms a critical part of the telomerase complex which maintains telomeres but is normally silenced in somatic cells. However, *TERT* gene expression is activated in ~90% of cancers, facilitating replicative immortality.[97] TERT activation in cancer cells can be achieved via mutations in the promoter region, or methylation of upstream CpG sites. The *TERT* hypermethylated oncological region (THOR) is a small genomic region containing 52 CpG sites immediately upstream of the core *TERT* gene promoter. Methylation of THOR is associated with gene expression, whereas unmethylated THOR prevents binding of repressive CTCF and is associated with gene silencing.[21,22] THOR methylation has been detected in a range of cancer types, including colon, ovarian, breast, lung, brain, prostate, bladder and blood cancers[21] (Table 1), making it an attractive therapeutic target. Targeted de-methylation of THOR using a modified CRISPR-dCas9 system has been demonstrated in breast cancer cells, and this was shown to result in a low-grade phenotype in cell line xenografts.[98] However, the degree of de-methylation using this approach was not optimal. Treatment of cancer cells pre-clinically with the global de-methylating agent 5′-aza-2′-deoxycytidine has also been shown to cause THOR hypomethylation and *TERT* gene silencing in many cases,[22,99] presenting another potential therapeutic avenue for cancers with THOR methylation.

How does aberrant DNA methylation occur in the first place?

Tumour suppressors are not generally silenced in normal tissues, precisely because they are important cellular gatekeepers. So, what goes wrong during human development or cell division that leads to this phenomenon? In some cases it appears that methylation is a critical early step in tumour formation,[100–102] but in other cases oncogenic drivers have been found to alter the epigenome, for example, activating mutations in *KRAS* in CRC and *IDH1* in glioma which drive the CpG island methylator phenotype (CIMP).[103,104]

It is interesting that certain tumour types are enriched for inactivation of certain tumour suppressors, whether by mutation or promoter methylation. For example, *BRCA1* methylation is frequently observed in cancer types where *BRCA1* mutations are also frequently observed.[25,105,106] This begs the question: is methylation of tumour suppressors a random somatic event occurring during development that simply promotes formation of certain cancers, or a directed event that reflects the silencing programme of the cell of origin.[107,108] The latter model is based on the idea that chromatin features of stem and progenitor cells may act as a 'blueprint' for methylation patterns that can be adopted by cancer cells, providing them with stem-like features.[107] There appears to be evidence for both models, depending on the gene and cancer context.

The CpG island methylator phenotype

In some cases, tumour suppressor methylation is associated with CIMP, where multiple tumour suppressor genes can be methylated concurrently. CIMP is primarily detected in colorectal and endometrial cancers.[109,110] The exact causes of CIMP are not fully understood,[111] but several factors have been described as potential contributors to its development. Certain genetic mutations or alterations have been proposed to lead to the development of CIMP due to their frequent observation in CIMP cancer types. The best example of this is association between *BRAF* and *KRAS* oncogene mutations in Colorectal Cancer (CRC) and CIMP.[103,112] There is evidence that activating *KRAS* mutations can drive CIMP via a transcriptional silencing pathway.[103] However, some recent work using ageing organoid models representing a CIMP epigenome showed that in the case of *BRAF*, CIMP is required for *BRAF* mutations to form cancer.[113] There is also evidence that *IDH* mutations can drive CIMP in glioma by modifying histones and increasing global DNA methylation.[104] Thus, it is possible that the order of CIMP *versus* oncogenic mutations may depend on the gene and disease context, although further studies are required to confirm this.

There are a variety of ways CIMP can develop and drive cancer. Age-related changes in DNA methylation patterns are well known,[100] and CIMP has been associated with older age in certain cancer types.[114] It is believed that cumulative exposure to various environmental factors over time and the gradual accumulation of epigenetic changes might contribute to age-related CIMP. Chronic inflammation has also been implicated in the development of CIMP in some cancers. Inflammatory processes can lead to the recruitment of immune cells and the release of cytokines, which can influence DNA methylation patterns and contribute to CIMP.[115] For example, inflammation caused by *Helicobacter Pylori* infection can lead to aberrant DNA methylation in gastric epithelial cells which ultimately drives gastric cancer formation.[116] Epstein–Barr virus infection, which is associated with a number of cancer types, including gastric, has also been linked to CIMP;[117,118] however, this mechanism may be via overexpression of DNMT1 following infection rather than inflammation.[117,119]

Although CIMP is always associated with methylation of tumour suppressor genes, tumour suppressor gene methylation is not always associated with CIMP. In fact, in some cases, methylation of a single tumour suppressor gene appears to be sufficient to drive cancer formation. For example, this is observed in cases of constitutional tumour suppressor gene methylation, which is described in the following section.

Constitutional methylation and cancer pre-disposition

Germline mutations in tumour suppressor genes are well-known drivers of carcinogenesis, and there is now a growing appreciation that aberrant methylation of tumour suppressors (i.e. epimutations) in normal tissues can also play a role in cancer formation and pre-disposition.[11,102,120] Constitutional methylation describes methylation of specific genes present in normal tissues, confined to one allele and present either in all cells or in mosaic form[101,102] (Figure 2). It most likely

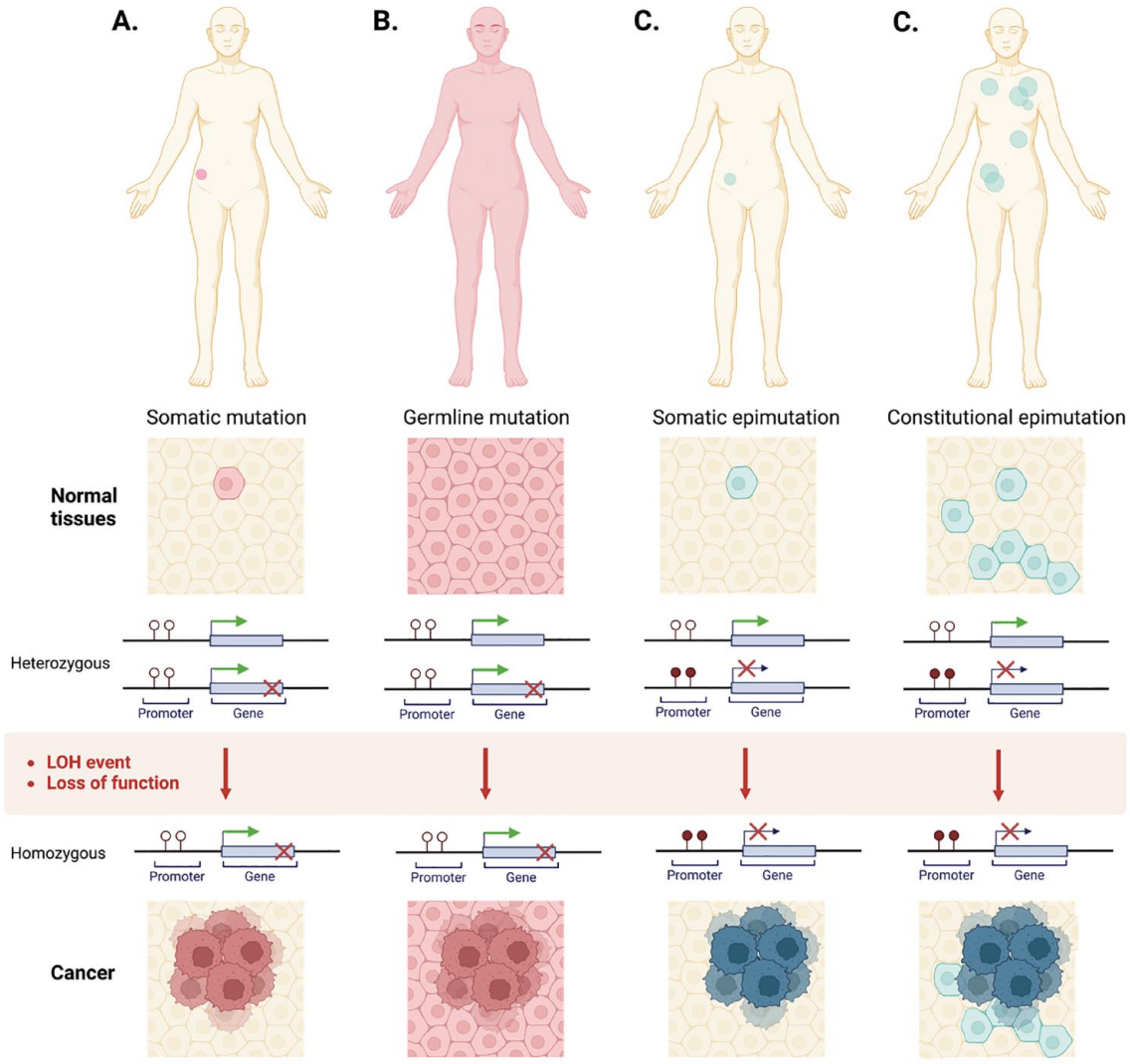

Figure 2. Cancer caused by somatic mutation, germline mutation or constitutional epimutation.
Three examples of a heterozygous tumour suppressor gene defect that becomes homozygous after a LOH event and drive cancer formation. (a) Somatic mutations can occur in individual cells at any point during life, inactivating one copy of the tumour suppressor in a given cell. If an additional LOH event occurs, then the wildtype copy of the gene is lost, so there are no functional copies remaining. (b) Germline mutations are inherited and affect only one copy of the gene in carriers but are present in all cells of the body. Such mutations can greatly increase the risk of cancer over an individual's lifetime. Like somatic mutations, the remaining wildtype copy of the gene can be lost by LOH, leading loss of gene function and cancer formation. (c) Epimutations can occur at any stage of development. In some cases, they occur as somatic events only found in tumour cells. (d) In other cases, they present as somatic mosaicism, or constitutional methylation, when they occur early in development (shown here). This means that multiple populations of cells within an individual will contain the epimutation. The earlier in development the epimutation occurs, the more cells/tissues of an individual will be affected. These epimutations silence the gene, but typically affect only one allele. The other allele can be lost by LOH, leading to on tumour suppressor gene copy that is fully silenced, and this can trigger carcinogenesis. Secondary epimutations are driven by a genetic event and can thus be heritable and behave as germline mutations as shown in (b); however, such epimutations are rare.
LOH, loss of heterozygosity.

arises early in development as a somatic event that then expands through different germ layers at varying frequencies and proportions, depending on the time and location of the event[102] (Figure 2). Constitutional epimutations in various genes have been described and linked to formation of various cancer types (reviewed in detail by[101,102,105,121]). Most epimutations develop independently of changes in the DNA sequence and are referred to as primary epimutations. In contrast, secondary epimutations are a consequence of a genetic change in a *cis* or *trans*-acting

factor and can thus be heritable if they occur in a germ cell. Secondary epimutations via in-*cis* genetic changes were initially described in the 1990s for the *FMR1* gene, which causes a neurodevelopmental disorder called Fragile X syndrome. Since then, such secondary constitutional epimutations have been described in other genes and for a range of additional disorders, including various cancers.[73,121,122] For example, a heterozygous c.-107A>T variant in the 5′ UTR of the *BRCA1* gene has been associated with *BRCA1* promoter methylation, and found to be dominantly inherited (present in all three germ layers) in two families affected by familial breast and ovarian cancer.[122] In cancer, tumour suppressor epimutations appear to rely on methylation of a single allele followed by inactivation of the wild-type allele, consistent with Knudson's two-hit model of tumour formation for tumour suppressor mutations[123,124] (Figure 2). Pathogenic germline tumour suppressor gene mutations are present soma-wide, and heritable secondary epimutations would be detected in the same way.[121] In contrast, primary epimutations are a somatic event and typically detected as an organism-wide mosaic pattern when arising early in development (Figure 2). Therefore, it is not surprising that constitutional epimutations are associated with increased cancer risk across multiple cancer types and related to a variety of tumour suppressor genes.[105] For example, an association has been observed between constitutional *BRCA1* promoter methylation and breast or ovarian cancers with *BRCA1* promoter methylation.[125,126] In both tumour types, *BRCA1* promoter methylation is mainly present in white blood cells of patients with tumours that have the same morphological characteristics as tumours of patients with a *BRCA1* mutation (*BRCA1*-like), implicating methylation as the mutagenic driver.[125,126] Indeed, *BRCA1* promoter methylation is rare outside of these two tumour types, suggesting that the methylation event indeed influences tumour initiation.[105,125–128]

Thus, constitutional methylation of tumour suppressors presents an alternative avenue to cancer formation, independent of CIMP. However, it is still not clear how epimutations arise during development in the first place, and then how these aberrant methylation marks are maintained in cells across generations of cell division. Perhaps the maintenance is a passive mechanism (e.g. DNMT1 simply continuing to replicate a methylation pattern that has formed at random), or some other marks or factors may actively instruct maintenance of this aberrant methylation.[107] It is also possible that both mechanisms are true, but relevant in different tumour suppressor genes or cancer contexts.

Impacts of tumour suppressor methylation on cancer therapy responses

Exploiting silencing of tumour suppressor genes in the clinic

In some cases, silencing of tumour suppressor genes by promoter methylation can have significant impacts on therapeutic responses. One example already discussed is methylation of *MLH1* or *MSH2* driving MMR deficiency and leading to sensitivity to immune checkpoint blockade. Another example is the impact of *BRCA1* and *RAD51C* methylation on PARPi responses in ovarian and breast cancers.[25,28,129]

BRCA1 and *RAD51C* are critical genes in the homologous recombination DNA repair pathway, and mutations in these genes have been shown to sensitize ovarian and breast cancer cells to PARPi.[130] Methylation of these tumour suppressors has been strongly associated with gene silencing, and genomic signatures associated with homologous recombination DNA repair deficiency (HRD), like *BRCA1/2* mutated cases.[28,81,131–134] It has been confirmed that me*BRCA1* and me*RAD51C* do indeed predict PARPi responses in ovarian cancer when in a homozygous state, that is, when all cellular gene copies harbour promoter methylation and are completely silenced, leading to HRD.[12,25,28] Me*BRCA1* has also been associated with good prognosis in TNBC patients.[26] However, homozygous me*BRCA1* or me*RAD51C* can be lost under platinum or PARPi pressure in breast and ovarian cancer, becoming either heterozygous or fully lost.[25,28,135] A single unmethylated gene copy of *BRCA1* or *RAD51C* is sufficient to restore HR DNA repair and drive PARPi/platinum resistance in ovarian cancers[25,28] (Figure 3), as is observed for heterozygous pathogenic mutations in these genes. Thus, monitoring for real-time quantitative methylation in cancers could help with clinical decision-making.

An additional example of this phenomenon is methylation of the tumour suppressor and DNA repair protein *MGMT* (O6-methylguanine–DNA methyltransferase) sensitizing Glioblastoma cells to treatment with alkylating agent temozolomide,[136] with its expression is associated with

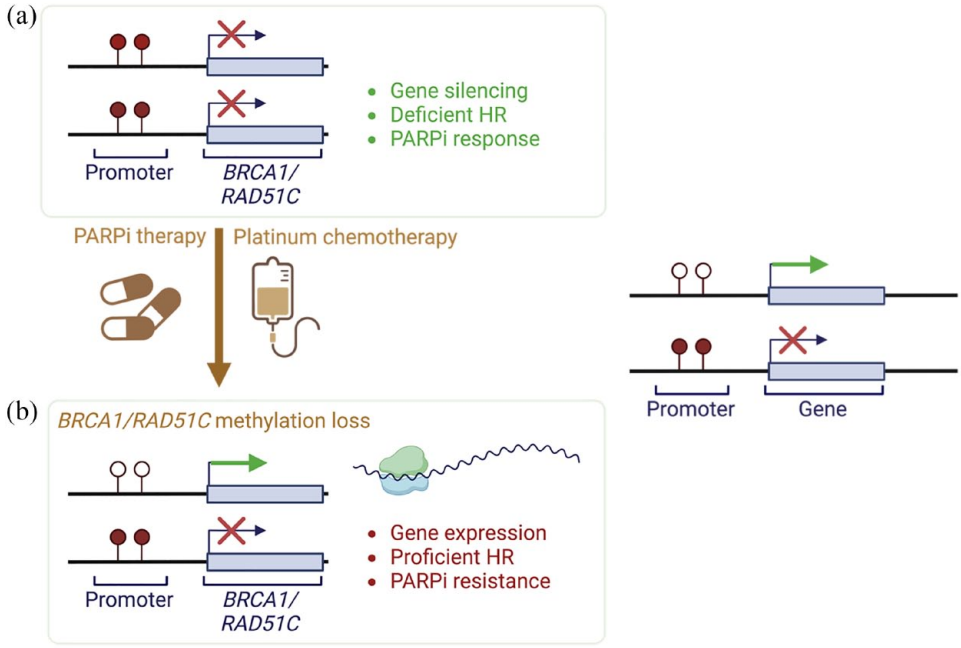

Figure 3. *BRCA1* or *RAD51C* methylation loss and gene re-expression can arise under treatment pressure in ovarian cancer, leading to therapeutic resistance.

temozolomide resistance.[137] Thus, the anti-therapeutic effects of de-methylation may occur in additional circumstances.

Demethylation as a therapeutic strategy

In the last decades, various epigenetic modulators have been developed and investigated in preclinical and clinical trials to target DNA hyper- and hypomethylation.[138] Preclinical research has clearly demonstrated the ability of demethylating therapies to decrease tumour suppressor promoter methylation and restored tumour suppressor gene expression, resulting in tumour cell reprogramming and, ultimately, cell death.[139–144] DNA methyltransferase (DNMT) inhibitors (DNMTi) directly impact DNA methylation at a global level within treated cells, whereas other epigenetic therapies, such as histone deacetylase inhibitors (HDACi) and enhancer of zeste homologue inhibitors (EZH2i), can have indirect effects on DNA methylation or gene expression, and are thus often explored in combination with DNMTi in cancers with dysregulated DNA methylation.

As a monotherapy, the most clinically validated epigenetic targeted therapies are DNMTi. 5-azacytidine (5-AZA) and 5-aza-2′deoxycytidine (decitabine). These are cytidine analogues that incorporate into DNA and inhibit DNMT activity by trapping it, leading to degradation by the proteasome and consequently resulting in hypomethylation during cell replication. 5-AZA and decitabine were approved by the Food and Drug Administration (FDA) in 2004 and 2006 for single agent therapy of myelodysplastic syndromes (MDS) and chronic myelomonocytic leukaemia (CMML).[145,146] The relevance of epigenetic treatment in haematological malignancies has been established for years and is reviewed in Santini *et al.*[147] However, 5-AZA and decitabine have several drawbacks: chemical instability, low bioavailability,[148,149] and off-target effects (primarily myelosuppression) due to non-selective inhibition.[150] Thus, their therapeutic use for solid tumours has been limited. To overcome these drawbacks, more stable nucleoside analogues, for example, SGI-110 (guadecitabine), and non-nucleoside compounds have been investigated. Furthermore, a highly potent DNMT1-selective small molecule inhibitor GSK3484862 has recently been developed.[151] In murine embryonic stem cells, this inhibitor caused promoter methylation loss and restored expression of the *VIM* gene (encoding vimentin), as well as a global decrease in DNA methylation levels with limited toxicity.[144,152] However, to date no clinical trials have been undertaken with the new selective non-nucleoside compounds.

The use of epigenetic modulators in multidrug combinational therapies has been shown to improve cancer treatments and potentially overcome drug resistance. Indeed, the synergy of epigenetic drugs and immunotherapies has recently emerged as a very promising field for cancer treatment.[153] For example, results of phase I and II studies have demonstrated that combining nucleoside-based DNMTi with either additional epigenetic drugs, immunotherapies or chemotherapies led to potential benefit in non-small lung cancer[154–156] and ovarian cancer.[157–159] The mechanisms reported to contribute to the synergistic effects of DNA hypomethylating and immune-targeting therapies include modulation of the tumour microenvironment,[160] direct activation of tumour-killing T cells[161] changes in immune checkpoint pathway expression[160,162,163] and generation of tumour neoantigens that reveal cancer cells to the immune system.[164,165]

It should be noted that in the context of me*BRCA1*/me*RAD51C* ovarian carcinomas, demethylating therapies, such as DNMT1 inhibitors, might be strategically detrimental for this group of patients, where tumour suppressor methylation is a positive biomarker for PARPi responses. This is less likely to be an issue for MMR pathway loss due to *MLH1* or *MSH2* silencing, as the immune therapies that exploit these defects rely on the existing DNA damage (MSI) caused by MMR loss, and not an active and continued silencing like that for the HR genes. Thus, the use of these agents in patients should be weighed against the benefits of tumour suppressor methylation that can be targeted by other therapies.

Methods for measuring DNA methylation

Most methods of detecting methylated DNA involve bisulphite conversion – a treatment of DNA that converts unmethylated cytosines in the genome to uracils, but leaves methylated cytosines unaltered. Following PCR, the converted cytosines are read as thymine; thus, methylated and unmethylated bases can be differentiated in a variety of assays (reviewed in detail in[166,167] and summarized in Table 2). However, additional new technologies are now emerging that can directly read DNA modifications from the native DNA (e.g. Oxford Nanopore long-read sequencing).[168]

When designing a test for methylation of a genomic region, it is important to consider not only which assay to use to answer a given question, but also which region of the gene/genome is most relevant. For example, if screening for tumour suppressor gene silencing via promoter hypermethylation, it is important that the region of the promoter being analysed is the critical portion that leads to gene silencing.[169,170] Furthermore, gene promoter hypermethylation does not necessarily imply that gene expression will be affected. For example, unlike *BRCA1* promoter methylation, *BRCA2* promoter methylation is not associated with gene silencing in epithelial ovarian cancer.[171,172] Thus, analysis of *BRCA2* promoter methylation will not yield useful information about DNA repair status of the cancer cells. If analysing methylation that is likely to be present at low levels (e.g. constitutional methylation, circulating tumour methylation in liquid biopsies or low purity tumour samples), it is best to opt for highly sensitive and quantitative assays, such as droplet digital PCR (ddPCR) or targeted bisulphite next-generation sequencing (bisNGS).[173] It is also important to consider whether specific information about individual CpG sites within a particular region is needed.[173] This can become important when assessing heterogeneous CpG methylation of a gene promoter, which is a common occurrence in cancer.[28,167] DNA methylation can also exist in a heterozygous state, leading to reduced gene expression, but incomplete gene silencing.[25,28] Measuring methylation zygosity can be challenging in patient tumour samples due to variable amounts of contaminating normal cells; however, highly quantitative methylation analysis combined with tumour purity and gene copy number estimates can assist with interpretation of results[25,28] (Figure 4).

In summary, measuring and interpreting promoter methylation in cancer is not always straightforward, and care should be taken when selecting technologies and designing assays or experiments to assess promoter methylation. In some instances, different assays can provide complementary information about methylation of a given region,[174] so it is always important to consider the question being posed, the genomic region being analysed and the purpose of the assay.

Tumour suppressor methylation in liquid biopsies

There is currently a growing interest in the use of cancer DNA methylation biomarkers for the screening, diagnosis and monitoring of cancer

Table 2. Summary of methods available for tumour suppressor methylation testing.

Method name	Input material	Method details	Advantages	Limitations
MS-PCR	Bisulphite DNA	Methylation-specific primers amplify a specific region, products visualized by gel electrophoresis	Fast, inexpensive and simple to perform	Not quantitative, potential PCR bias
MethylLight	Bisulphite DNA	DNA methylation measured a fluorescent probe to measure MS-PCR amplification in real-time	Makes MS-PCR quantitative, simple and inexpensive	Only quantitative for homogeneous samples, potential PCR bias
SMART-MSP	Bisulphite DNA	Probe-free MS-PCR assay with evaluation by HRM	Quantitative, can detect heterogeneous methylation	Potential PCR bias, no individual CpG information
MS-SSCA	Bisulphite DNA	PCR of bisulphite DNA followed by heat denaturation and electrophoresis (SSCA)	Sensitive to 5–10%, can detect various epialleles, inexpensive	Semi-quantitative, no individual CpG information
MS-DGGE	Bisulphite DNA	PCR of bisulphite DNA followed by denaturing gradient gel electrophoresis (DGGE)	Can detect various epialleles, inexpensive	Semi-quantitative, no individual CpG information
MS-DHPLC	Bisulphite DNA	PCR of bisulphite DNA followed by denaturing high-performance liquid chromatography	Fast, quantitative, sensitive, cost effective	No individual CpG information
MS-HRM	Bisulphite DNA	DNA binding dye monitors different melting profiles of PCR products	Fast, quantitative in some cases, sensitive, cost effective	Only semi-quantitative in some cases, no individual CpG information
Bisulphite Pyrosequencing	Bisulphite DNA	PCR of bisulphite DNA followed by pyrosequencing	Quantitative, sensitive, cost effective, can be targeted or whole-genome	Shorter sequences analysed, only average methylation level per CpG position
Sequenom MassARRAY (EpiTYPER)	Bisulphite DNA	PCR of bisulphite DNA followed by base-specific cleavage and MALDI-TOF	High-throughput, (semi-) quantitative analysis of multiple CpG sites	Only average methylation level per CpG position
MS-SNuPE	Bisulphite DNA	PCR of bisulphite DNA followed by primer extension, detection systems include radioactivity, chromatogram or fluorescence	Quantitative, multiple detection systems	Limited access to certain CpG sites, usually only one CpG per experiment
COBRA	Bisulphite DNA	PCR of bisulphite DNA followed by digestion with restriction endonucleases and gel electrophoresis	Fast, inexpensive and simple to perform	Qualitative to semiquantitative results per CpG, limited CpG sites per enzyme
Digital MS-HRM	Bisulphite DNA	Like MS-HRM but using limiting amplicon dilutions followed by sequencing	Melting profiles of individual epialleles in heterogeneous samples	Does not provide individual CpG information
Digital MethylLight	Bisulphite DNA	Like MethylLight but PCR amplification from single templates	Reduced PCR bias	Still not ideal for heterogeneous samples, no information on epialleles
Targeted bisNGS	Bisulphite DNA	PCR of bisulphite DNA using primers with NGS adaptors followed by bisNGS (e.g. on Illumina platform)	Highly quantitative, simple, relatively inexpensive, information on all CpG sites and epialleles, NGS multiplexing of samples/PCR product	Amplicons limited to ~300–500 bp, PCR multiplexing not tested yet

(Continued)

Table 2. (Continued)

Method name	Input material	Method details	Advantages	Limitations
Illumina methylation arrays	Bisulphite DNA	Bisulphite DNA is run on a microarray chip	Simple, relatively cost effective, genome-wide, popular option	Only average methylation level per CpG position
Whole genome bisulphite sequencing	Bisulphite DNA	Bisulphite DNA is amplified and sequenced	Genome-wide, CpG and epiallele resolution	Expensive, computationally intensive
RRBS	Bisulphite DNA	Methylation-insensitive restriction enzymes digest DNA, enriching for CpG regions in bisulphite DNA, followed by sequencing	Relatively inexpensive, higher coverage than whole genome bisNGS	Some CpG sites might be missed
Oxford Nanopore Sequencing	Native DNA	Targeted capture with nanopre sequencing, or no capture and adaptive sampling protocol for region of interest	No bisulphite conversion or PCR bias, long reads for phasing of larger regions	Expensive, platform still being optimized
MSRE/MRE-seq	Native DNA	Unmethylated DNA digested by restriction enzymes, methylated DNA amplified and sequenced	No bisulphite conversion bias	Bisulphite DNA may also be digested, limited coverage
MeDip	Native DNA	Methylated DNA is enriched by immunoprecipitation, followed by sequencing or microarray analysis	No restriction enzyme or PCR bias	CpG density can confound enrichment, uneven coverage

A summary of reported methods that can be used to detect or measure methylation of tumour suppressor promoters.
bisNGS, bisulphite next-generation sequencing; COBRA, combined bisulphite restriction analysis; DHPLC, methylation-specific denaturing high-performance liquid chromatography; HRM, high-resolution melt; MALDI-TOF, MALDI coupled time-of-flight mass spectrometry; MeDIP, methylated DNA immunoprecipitation; MS-DGGE, methylation-specific denaturing gradient gel electrophoresis; MS-MS-HRM, methylation-specific high-resolution melt; MS-PCR, methylation specific PCR; MS-SSCA, methylation-specific single-strand conformation analysis; MS-SNuPE, methylation sensitive-single nucleotide primer extension; MSRE/MRE-Seq or Methyl-seq, methylation sensitive restriction enzyme sequencing; RRBS, reduced-representation bisulphite sequencing; SMART-MSP, sensitive melting analysis after real time-MS-PCR.[166–168]

using blood samples from patients. Indeed, blood tests appear to be preferred by patients when given a choice of invasive and even other non-invasive tests.[175] Most of these tests are based on detection of circulating tumour DNA (ctDNA), tumour proteins, exosomes or circulating tumour cells and present with multiple advantages over conventional tissue biopsies.[176] Circulating tumour DNA (ctDNA) is released into the bloodstream by tumour cells, or circulating tumour cells, through processes like apoptosis, necrosis and active secretion. It can provide valuable genetic and epigenetic information about the tumour, such as mutations, copy number variations, and DNA methylation changes. These, in turn, can be used for non-invasive cancer screening, diagnosis, prognosis and disease monitoring.[177–179]

CtDNA methylation is a relatively stable chemical modification that can be detected in degraded and poor-quality DNA samples, like patient plasma or formalin-fixed samples. Given that certain DNA methylation changes are also cancer-specific, and these changes can occur early in cancer development, there has been growing use of DNA methylation as a cancer biomarker in liquid biopsies.[180] CtDNA methylation in liquid biopsies can be analysed as either a single region (e.g. a single gene promoter),[181–184] as a panel of loci (e.g. multiple gene promoters)[185–189] or using genome-wide epigenetic signatures.[181,190,191] Some of these approaches have been FDA approved. For example, Epi proColon [available from: https://www.epiprocolon.com/us/.] is an FDA approved kit for the detection of *SEPT9* DNA methylation in serum samples. This test is based on real-time PCR of bisulphite converted ctDNA and has demonstrated an overall sensitivity of 90% and specificity of 88% for detecting CRC at all stages in a retrospective cohort of patients.[192] Although the Epi proColon test relies on a methylation of a single gene, other tests analysing multiple genes or global epigenetic signatures in ctDNA also show great promise. An advantage of these types of tests is that they can potentially detect multiple cancer types in a single test, making them ideal tools for multi-cancer early detection (MCED). The methylation-based PanSeer assay (from Singlera Genomics; https://

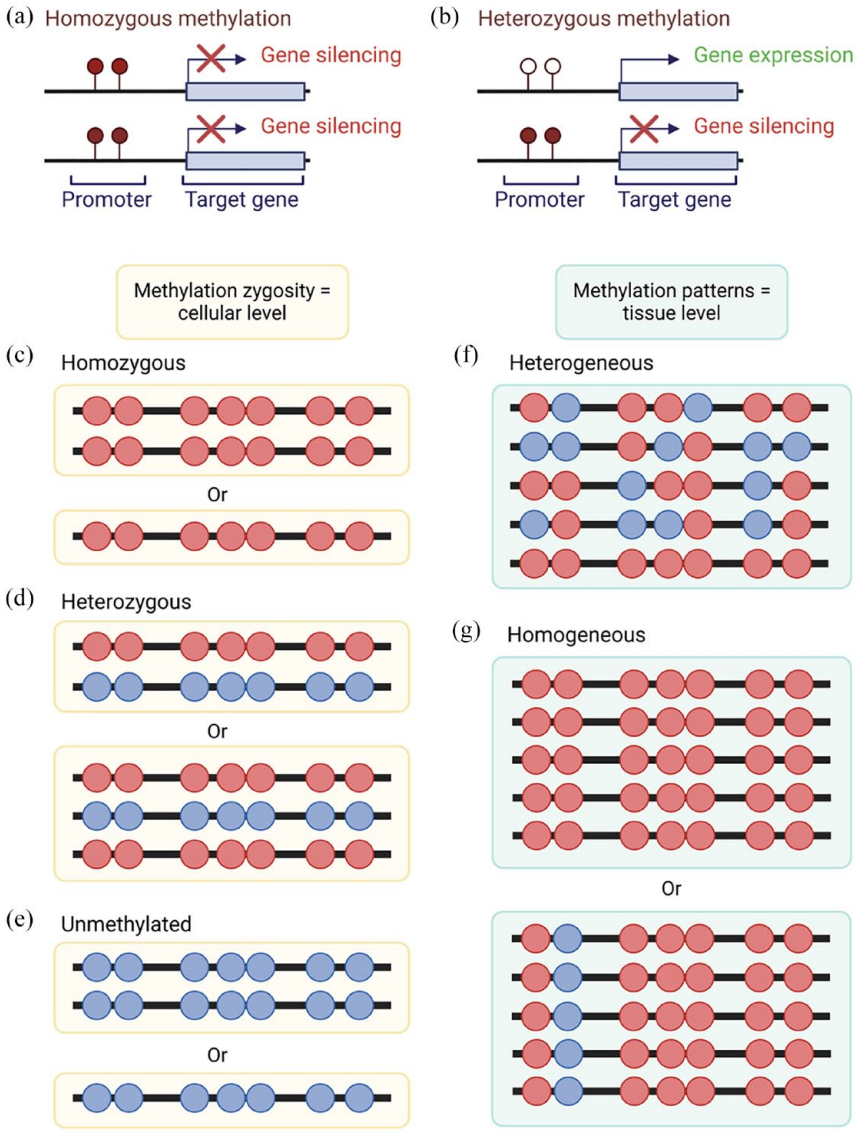

Figure 4. Definitions of promoter methylation states in tumours. (a) For a tumour suppressor to be fully silenced, the critical promoter CpG sites of all gene copies in a cell or tissue must be methylated. (b) If a single gene copy loses its promoter methylation, the gene product can be expressed and regain function. (c) Methylation zygosity describes combinations of epialleles present at the cellular level. 'Homozygous methylation' is when all gene copies in a cell have fully/highly methylated promoters (critical CpG sites are methylated) and the given gene is silenced. This includes aneuploid cases where one allele has been lost due and the remaining hemizygous allele is methylated. (d) 'Heterozygous methylation' describes mixtures of fully/highly methylated and unmethylated epialleles coexisting within each cell. In these cells, gene expression is active due to the presence of fully unmethylated epialleles, despite the presence of fully methylated epialleles. (e) Cells with no methylated epialleles have full gene expression. In the case of tumour suppressor genes, this is the normal state of non-cancer cells. (f) Methylation patterns describe epiallele diversity at the tumour/tissue level. 'Heterogeneous methylation' describes a highly heterogeneous mixture of epialleles with various CpG methylation patterns present in a tumour sample. (g) 'Homogeneous methylation' describes a homogeneous mixture of fully or highly methylated epialleles, which are dominated by one particular CpG methylation pattern in a tissue sample. This can include samples with homozygous methylation within their cells.

singleraoncology.com/) interrogates 595 regions at high sequencing depth using their proprietary MethylTitan platform and was shown to detect five common cancer types (stomach, oesophagus, colorectum, lung or liver) in 88% of post-diagnosis patients with a specificity of 96%. PanSeer also

detected cancer in 95% of asymptomatic individuals who were later diagnosed, though longitudinal confirmation is needed.[187] The PDACatch assay from Singlera is based on the same technology as the PanSeer and has recently been approved by the FDA for identification of pancreatic ductal adenocarcinoma (PDAC) in individuals at high risk for the disease and outperforms existing PDAC blood markers in terms of sensitivity.[188]

The Galleri test (from GRAIL; https://grail.com/galleri-test/), in contrast, analyses > 1 million methylation sites in cell-free DNA fragments to detect changes in global methylation patterns that could indicate cancer presence.[190,191] This test was developed based on findings from the Circulating Cell-free Genome Atlas study (CCGA; NCT02889978) showing that whole genome bisulphite sequencing outperformed WGS and targeted sequencing of short variants/indels in terms of cancer detection sensitivity, and that machine-learning classifiers could be used to detect cancer and predict cancer signal origin.[193] The resulting test was found to have a 99.3% specificity for detecting cancer[193] and was also able to predict cancer signal origin with 88% accuracy.[194] The test can detect a methylation pattern common to 50 cancer types, making it ideal for general cancer screening.[190] Validation of prospective patient cohorts is also ongoing in the following studies: PATHFINDER (NCT04241796) STRIVE (NCT03085888) and SUMMIT (NCT03934866), and several successful clinical case reports have been recently reported.[195,196] The results reported from these studies so far are extremely exciting, for example interim results of PATHFINDER demonstrated cancer signal in 1.5% (62/4033) of individuals screened, with 40/62 of these having reached diagnostic resolution to date.[197] Sensitive and accurate pan-cancer liquid biopsies would have a massive clinical impact by identifying cancer in patients early (improving survival outcomes) and reducing unnecessary invasive testing in healthy people.

It should be noted, that the presence of ctDNA is different to constitutional methylation of a particular tumour suppressor. Although ctDNA is tumour derived and may represent early or late stage cancer, constitutional methylation is derived from normal cells in the body and may only represent an increased risk of developing a cancer. Constitutional methylation may, thus, be a confounding factor in the detection of methylated ctDNA in liquid biopsies focussed on a single gene/region, potentially causing false positive results in patients. This should be a consideration when designing and interpreting results of such tests.

Conclusion

There is a growing appreciation of the impacts of epigenetic changes in the development and progression of cancer.[15] Indeed, 'Non-mutational Epigenetic Reprogramming' has recently been added to the Hallmarks of Cancer originally described by Hanahan and Weinberg.[198] Promoter methylation of various genes can have prognostic implications across many cancer types. Interestingly, tumour suppressor genes that are frequently silenced by methylation in certain cancer types also tend to be frequently inactivated by mutations in the same cancer types. This suggests that, like mutations, epimutations may be selected for during oncogenesis in a tissue and pathway specific manner. Amongst other examples of therapeutic implications, evaluation of methylation of *BRCA1*/*RAD51C* and *MLH1*/*MSH2* promoters can predict response to PARP and immune checkpoint inhibitors respectively. In the case of PARPi treatment of ovarian cancers with *BRCA1* or *RAD51C* methylation, however, tumour suppressor gene methylation is a therapeutic target that can be lost under treatment pressure.[25,28,129] Thus, monitoring of methylation using appropriately designed assays is critical for providing the best guidance for clinical decision-making. Indeed, we have highlighted the importance of appropriate design and selection of assays for measuring methylation, as each platform has its own advantages and disadvantages. With advances in DNA sequencing technologies, it is now possible to detect low levels of DNA methylation in tissue and blood samples. Identification of methylation in ctDNA from non-invasive liquid biopsies is opening exciting opportunities for early detection of cancer, both for specific cancer types and as a pan-cancer screening tool. Multiple assays are currently being trialled in patients, and preliminary reports have been promising.

There are still many important questions remaining. For example, in cases where tumour suppressor gene methylation is a therapeutic target, are there ways in which this methylation could be stabilized to prevent onset of therapeutic resistance? Also, could we prevent epigenetic silencing of tumour suppressor genes in order to avert cancer

initiation? An improved understanding of how methylation develops and is maintained in cancer cells could provide answers to these and other critical questions in the future.

Declarations

Disclaimer

Clare Scott is an Editorial Board Member of Therapeutic Advances in Medical Oncology and an author of this paper; therefore, the peer review process was managed by alternative members of the board and Clare Scott has not been involved in the decision-making process.

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Author contributions

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Competing interests

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