Methylation of DNA repair genes and the efficacy of DNA targeted anticancer treatment

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Abstract

Cancer is considered both a genetic and epigenetic disease. The best studied epigenetic mechanism in carcinogenesis is DNA methylation, a mechanism which inactivates tumor suppressor genes by methylating their promoters. A subclass of tumor suppressor genes that are often methylated in the process of carcinogenesis are the DNA repair genes. Accumulation of DNA damage is associated with cancer and thus inactivation of DNA repair genes promotes cancer formation. Methylation of DNA repair genes seems to be unique compared to the methylation of other tumor suppressor genes in that it not only promotes tumorigenesis, but at the same time influences the sensitivity of tumors to chemotherapies that are based on agents that damage DNA to kill cancer cells. This literature study summarizes the current knowledge regarding epigenetic inactivation of DNA repair genes and the subsequent progress that has been made coping with the acquired chemotherapy resistances.

Keywords: DNA repair, methylation, cancer, MGMT, BRCA1, MLH1

Introduction

Over the last decade, research on epigenetic events has increased as they play an important role in many cellular processes, including tumorigenesis. These epigenetic events refer to processes that functionally modify the genome without changing the actual nucleotide sequence, such as chromatin remodeling, histone modification, non-coding RNA, DNA acetylation and DNA methylation. DNA methylation can be considered one of the best studied epigenetic events, playing an essential role in (long-term) silencing of genes [1]. One of the reasons epigenetics has gained a lot of interest in cancer research in particular, is that these epigenetic modifications are not considered irreversible [2] and as such malignant cells could potentially be reverted back to normal healthy cells. DNA methylation not only plays a role in tumorigenesis, but also in the efficacy of anticancer drugs. This review focuses on the role of DNA methylation in the activity of DNA repair enzymes, which normally repair DNA damage caused by DNA directed anticancer agents.

Review

DNA methylation in cancer

The ability to silence genes explains the crucial role that DNA methylation has in X-chromosome inactivation [3], genomic imprinting [4], regulating gene expression as well as suppressing

transposons and repetitive elements (i.e., LINE) [5]. Methylation of DNA occurs by the covalent addition of a methyl (-CH₃) group to a cytosine of a cytosine-phosphate-guanine (CpG) dinucleotide. Clusters of these CpG dinucleotides are located in promoter regions of genes and are called CpG islands. The exact requirement for a DNA region to be defined as a CpG island is a length of at least 500 bp of which ≥55% consists of GC and a ratio of observed and expected CpG >0.65 [6]. DNA methylation of these CpG islands in promoter regions silences the genes to which these particular promoter regions belong by either recruiting additional silencing associated proteins and/or lowering the accessibility of the DNA for transcription factors (TFs) [7] (Figure 1).

DNA methylation is carried out by DNA methylation transferases (DNMTs). There are 5 DNMT isoforms: DNMT1, DNMT2, DNMT3a, DNMT3b and DNMT3L; but of which only DNMT1, DNMT3a and DNMT3b actually methylate DNA. DNMT3a and DNMT3b establish *de novo* DNA methylation patterns, important during embryogenesis [8]. Functionally, DNMT1 differs in that its role is to maintain the established DNA methylation pattern through cell division and thus DNA replication [9]. This is done by recognition of hemi-methylated DNA by UHRF1 and directing DNMT1 towards methylating the corresponding cytosine in the newly synthesized DNA strand [10].

For a cell to develop and function normally, proper DNA methylation is critical. Therefore, any aberrations in this event can lead to diseases, including cancer. Indeed, DNA of cancer cells has a different methylation pattern than that of normal cells. In general, a global hypomethylation of DNA can be observed in cancer cells. Although rather uncommon, this can lead to activation of previously silenced oncogenes, but also contributes to genomic instability and mutagenesis by reactivation of transposable elements which then can integrate at random sites. However, local hypermethylation also takes place in the transformation into a cancer cell, namely the methylation of promoter regions of so called tumor suppressor genes (TSG) [11]. These are genes that play a role in the suppression of tumorigenesis in normal cells.

The genes that are hypermethylated in malignant cells often have various but also important cellular functions. For example, genes that are related to cell cycle regulation such as *P16^{INK4A}* and *P15^{INK4B}* are silenced in many cancers due to DNA methylation enabling uninhibited proliferation (e.g., [12]). Other genes often hypermethylated, and thus silenced, in cancer can be involved in: cell adhesion, such as E-cadherin and H-cadherin, enabling invasion and/or metastasis of the tumor (e.g., [13]); apoptosis, such as *DAPK1*, enabling resistance to cell death (e.g., [14]); and finally genes associated with DNA repair processes are also often hypermethylated in tumors, thus suggesting that epigenetic events may promote classical genetic alterations such as mutations.

DNA repair pathways and their genes

As described above, genes involved in the repair of damaged DNA are an important class of tumor suppressor genes. The pathways in which their products act on DNA damage are part of what is considered the DNA damage response (DDR). This DDR generally protects cells against genomic instability, which is considered a characteristic of tumorigenesis [15]. As such, silencing of these genes via hypermethylation is a common occurrence in cancers. For this reason it is important to realize that the efficacy of therapies based on damaging

DNA to successfully kill cancer cells, such as radiotherapy and certain chemotherapies is largely dependent on inhibition of these DNA repair pathways [16]. There are multiple different DNA repair pathways available and the involvement of a specific pathway will depend on the type of DNA lesion that needs to be repaired.

Base excision repair

Damaged bases are excised and replaced by enzymes belonging to the base excision repair (BER) pathway. These lesions generally arise due to oxidative damage caused by reactive oxygen species (ROS). If damaged bases are ignored there is an increased chance in mispairing of the bases, potentially causing mutations. The most frequent oxidations of bases are 8-oxoguanine (8-oxoG) and 5-hydroxycytosine, which mispair with adenine and thymine respectively [17,18]. However, BER is also able to repair alkylated bases, namely 3-methyladenine and 7-methylguanine. The first step of BER is the removal of the damaged bases by glycosylases, resulting in apurinic or apyrimidinic (AP) sites, after which endonucleases create a single strand break (SSB). This will then be processed by DNA polymerases during either short-patch (single nucleotide replacement) or long-patch BER (2-10 nucleotides replacement). Pol β is the main DNA polymerase that catalyzes short-patch BER, with pol λ being able to take over in its absence [19], while DNA synthesis during long-patch BER is mediated by pol δ and pol ϵ . Pol δ and ϵ perform displacing synthesis, generating a "displaced" DNA flap which is removed by FEN-1. The seam between new DNA and the already existing DNA strand is sealed by DNA ligase III and the XRCC1 protein in short-patch BER, while this is catalyzed by DNA ligase I in long-patch BER (Figure 2A).

Nucleotide excision repair

While the BER pathway can repair specific non-bulky lesions, the nucleotide excision repair (NER) pathway can remove bulky DNA adducts such as thymine dimers, often induced by UV radiation [20]. These adducts are recognized by several proteins such as DDB, XPA and the XPC-Rad23B complex. After damage recognition, the DNA helix is unwound by Transcription factor II H (TFIIH) and subsequently two incisions are made, one at each of 3' and 5' end of the site. The 3' end incision is made by the XPG protein, while the 5' end incision is performed by the heterodimeric XPF-ERCC1 protein [21]. This leads to the removal of 25-30 nucleotides. The created gap is then filled with newly synthesized DNA by DNA polymerases δ and ϵ after which the nicks are ligated by either DNA ligase I or DNA ligase III-XRCC1 (Figure 2B).

Mismatch repair

Mismatch repair (MMR) of DNA is specifically aimed at, as the name implies, the repair of mismatched base pairs caused by either erroneous base insertions, deletions or mis-incorporations during DNA replication. The mismatch is

recognized by heterodimeric proteins MSH2-MSH6 and MSH2-MSH3. Next a nick is created in the strand near the mismatch lesion by the heterodimeric protein and subsequently a segment of single strand DNA containing the mismatch is excised by exonuclease 1 (EXO1). The resulting single strand gap is then repaired by DNA polymerase δ and finally sealed by DNA ligase [22] (Figure 3A).

Direct repair

Direct reversal can be considered the simplest form of DNA repair. It is considered direct in that it does not involve breaking the phosphodiester backbone of DNA, removing nucleotides and replacing them with newly synthesized DNA. DNA damage in the form of methylation of guanine at the O⁶-position can be removed by the O⁶-methylguanine DNA methyltransferase (MGMT) protein [23]. MGMT also has the ability to remove other alkylations of the O⁶-position of guanine [23] (Figure 3B). Unrepaired O⁶-methylguanine lesions can lead to mutagenesis as distorted pairing of guanine with cytosine or thymidine leads to G:C to A:T transitions on replication [24], triggering cytotoxicity and apoptosis if left unrepaired [25]. The removal of the methyl group from guanine by MGMT as the transferase and acceptor is a stoichiometric reaction. This means a single MGMT protein can only be used for a single removal of one methyl group from the O⁶ position of

guanine. The conformational change of the MGMT protein by accepting the methyl group will subsequently target it for degradation through the ubiquitin proteolytic pathway [26].

Non-homologous end joining

Repair of double strand breaks (DSB), in which both strands of the double helix are damaged, can be accomplished by two different pathways of which non-homologous end joining (NHEJ) is one. DSBs are more difficult to repair than SSBs and as such require a more elaborate method of repair. DSBs are also inherently more cytotoxic than any other DNA damage. NHEJ is an error-prone pathway as it uses no homology (such as the sister chromatid) or very little for its repair function, potentially causing deletions and/or insertions [27]. The first protein to be recruited and bound to a DSB lesion is the Ku heterodimer and serves as a docking station for other additional proteins required for NHEJ. The Artemis/DNA-PKcs complex, of which the Artemis protein functions as an endonuclease, excises any damaged nucleotides at either DNA end if necessary [28,29]. Polymerases μ and λ are then able to fill the gaps at both the 5'-end and 3'-end overhang with newly synthesized DNA. Ligase, as the final step, is executed by a complex consisting of XLF, XRCC4, and DNA Ligase IV [30] (Figure 4A).

Homologous recombination repair

The other pathway able to repair DSB lesions is the homologous recombination (HR) pathway. Unlike NHEJ, HR uses an undamaged DNA template of either a sister chromatid or

homologous chromosome to repair the damaged strands, leading to reestablishment of the original sequence relatively error-free [31]. After the occurrence of a DSB, the MRN complex consisting of the proteins Mre11, Rad50 and Nbs1, binds to both ends of the break which are subsequently processed into 3' ssDNA by CtIP-mediated nuclease activity [32,33]. The ssDNA end is coated with DNA replication protein A (RPA), after which RAD51 nucleoprotein filament is assembled, which replaces the RPA-coated ssDNA ends, performs homology sequence searching, mediates strand invasion and promotes DNA strand exchange [34,35]. Once the homologous DNA has been identified, the damaged DNA strand invades the undamaged DNA duplex with the help of Rad54 in a process referred to as DNA strand exchange [36]. A DNA polymerase then extends the 3' end of the invading strand and subsequent ligation

by DNA Ligase I yields a hetero-duplexed DNA structure. Completing the recombination requires the separation of the recombining partners by the enzyme Resolvase. This recombination intermediate is resolved and the precise, error-free correction of the DSB is completed (Figure 4B).

DNA repair and cancers

As previously mentioned the various DNA repair pathways are crucial in protecting cells against genomic instability, a characteristic of cancer development. Aberrations in the genes that are part of these pathways increase the susceptibility to cancer growth. This explains why many common cancers often possess aberrations in specific DNA repair pathways (see Table 1). For example, both breast and ovarian cancers often contain mutations in BRCA1 which is an important protein of the HRR pathway [37], while the MGMT gene of the direct repair pathway is often hypermethylated in glioblastomas. Susceptibility to hereditary nonpolyposis colorectal cancer (HNPCC) can in many cases be attributed to dysfunctional MLH1 and MSH2 proteins in the MMR pathway [38] and patients with a deficiency in Artemis, an important factor in NHEJ, have a predisposition to lymphomas [39]. Reduced expression of the ERCC1 protein in the NER pathway is associated with an increased risk of squamous cell carcinoma of the head and neck (SCCHN) due to the inability to sufficiently repair genetic damage induced by tobacco carcinogens such as benzo[a]pyrene diol epoxide [40]. These are only a few examples of the many cancers in which the aberration of DNA repair pathways is an important factor in their development.

Table 1. Examples of common DNA repair aberrations in cancer.

Protein	Pathway	Cancer type
BRCA1	HRR	Breast and ovarian cancer
MGMT	Direct repair	Glioblastoma
MLH1 and MSH2	MMR	HNPCC
Artemis	NHEJ	Lymphoma
ERCC1	NER	SCCHN

Alkylating and alkylating-like agents

Anti-cancer drugs that act by inducing lethal levels of DNA damage are often based on the process of alkylation. Alkylation is the transfer of an alkyl group (C_nH_{2n+1}) from one molecule to another. In the case of these so-called alkylating agents, the alkyl group is transferred to the 7-N position of the guanine base of DNA. Alkylating-like agents cause similar effects through a comparable process, but do not contain an alkyl group. Instead they achieve similar results by transferring other groups to the 7N guanine position, such as platinum-based drugs. In both cases however, this can lead to cross-linking of the guanine bases of the two strands contained in the DNA double-helix, effectively making it impossible for the two strands to unwind and separate, in turn preventing the

replication of DNA and thus preventing proliferation. The efficiency of these agents is however based on the assumption that DNA repair is down regulated in cancer cells. Indeed, in the case of many tumors the DNA repair has been shut down in an early stage to increase the levels of mutagenesis and genetic instability, considered one of the important characteristics of malignant transformation of cells [41]. However, it is not necessarily required and many tumors have a relatively intact DNA repair mechanism, potentially hindering the function of these alkylating agents, leading to resistance. Since the discovery of nitrogen mustards and cisplatin, examples of the very first drugs in the class of alkylating/alkylating-like agents, a great number and wide variety of new drugs have been developed in this class for a large number of different cancers.

Methylation status of DNA repair genes

As mentioned above, for alkylating/alkylating-like agents to exert their anti-cancer capabilities they require the inactivation of the DNA repair response in the cancer cells. Silencing of the genes crucial in these pathways can be accomplished by either inactivating mutations or, as described previously, by methylation of the gene promoter. Methylation patterns vary widely between normal cells and cancer cells and DNA repair genes, which are considered tumor suppressor genes (TSGs), are often methylated in varying cancer types (see Table 2). Due to this, the methylation status of certain critical DNA repair genes can be decisive whether a chemotherapy with alkylating agents will have their desired effect. This is important as a chemotherapy can have severe side effects and treating a patient who has a tumor resistant to that particular therapy will lead to an unnecessary burden for the patient and wastes both (critical) time and resources. Depending on which DNA repair gene is either methylated or unmethylated, the therapy to which resistance is obtained varies. So far, the majority of candidate predictive biomarkers belong to the group of DNA repair genes. These are often epigenetically altered in cancer and can significantly influence the response to chemotherapy [42]. The DNA repair genes of which the methylation status is considered to have a strong predictive potential will be discussed below.

MGMT methylation

MGMT is a monomeric enzyme and was first discovered in bacteria in 1977 [43]. MGMT is the only and thus crucial enzyme in the direct reversal pathway. This pathway differs from the other described DNA repair pathways in that it does not create incisions in the phosphodiester backbone to repair the DNA strand. Its main repair function is the removal of O⁶-methylguanine adducts, although it is also able to remove other alkyl adducts. As such MGMT prevents the genotoxic effect of O⁶-alkylguanine adducts produced by exogenous and endogenous alkylators in cells. As previously mentioned, the removal of adducts by MGMT is considered stoichiometric

Table 2. Examples of methylated DNA repair genes in cancer.

Repair pathway	Gene	Cancer type	Reference
Base excision repair	MBD4	Multiple myeloma	[98]
		Colorectal cancer	[99]
		Ovarian cancer	[99]
Nucleotide excision repair	TDG	Multiple myeloma	[98]
		Papillary thyroid cancer	[100]
	OGG1	Bladder cancer	[101]
		Lung cancer	[102]
Mismatch repair	XPC	Multiple myeloma	[103]
		Glioma	[93,94]
	ERCC1	Sporadic colorectal cancer	[104]
		Colon cancer	[105]
Direct repair	MLH1	Sporadic endometrial cancer	[106]
		Gastric cancer	[107,108]
		Ovarian cancer	[109,110]
		Acute myeloid leukemia	[111]
		Non-small cell lung cancer	[112]
		Head and neck squamous cell carcinoma	[113,114]
		Oral squamous cell carcinoma	[115]
		Non-small cell lung cancer	[112]
		Oral squamous cell carcinoma	[115]
		Ovarian cancer	[110]
Non-homologous end joining	MSH2	Colon cancer	[116]
		Glioblastoma	[53]
		Gastric carcinoma	[117]
		Non-small cell lung cancer	[118]
		Small cell lung cancer	[54]
		Head and neck squamous cell carcinoma	[119]
		Cervical cancer	[120]
Homologous recombination	MGMT	Non-small cell lung cancer	[121]
		Breast cancer	[75]
		Non-small cell lung cancer	[121]
		Ovarian cancer	[122]
		Uterine leiomyosarcoma	[123]
		Gastric cancer	[124]
	BRCA1	Bladder cancer	[125]

and thus the number of adducts removed is limited by the amount of MGMT present in the cells and the rate at which it is synthesized.

MGMT expression and resistance to alkylating agents

MGMT is ubiquitously expressed in normal human tissues, but expression levels differ widely between tissues. Many human

tumors express MGMT as well, but a fraction of gliomas lacks any detectable expression of MGMT as observed in multiple studies [44-46]. Silencing of genes in tumors is often achieved by hypermethylation of CpG islands in their promoter regions. Indeed, the promoter region of MGMT has been found to be methylated in human tumors lacking its expression [47,48]. It is well established that expression of MGMT leads to resistance against alkylating agents such as temozolomide (TMZ), streptozotocin, bis-chloroethylnitrosourea (BCNU, Carmustine) and CCNU in glioma cell lines [49-51] and xenografts [52]. Glioma patients with methylated MGMT promoter were more sensitive to BCNU than those with unmethylated MGMT, showing a 64% and 4% response rate (RR) respectively [53]. A phase II trial of TMZ in patients with small cell lung cancer showed that patients with a methylated MGMT promoter had a better response compared to those with unmethylated MGMT (38% vs. 7%) [54]. Especially TMZ is an important alkylating agent for chemotherapeutic treatment of patients suffering both high- and low grade brain tumors. TMZ is an alkylating agent which adds a methyl group to guanine on the O⁶ position. These O⁶-methylguanine adducts induces cytotoxicity by causing DNA polymerases to insert a thymidine opposite the adducts during DNA replication instead of a proper cytidine. This is an erroneous pairing of nucleotides and as such recruits MMR. The MMR excises a short strand of newly synthesized DNA including the erroneous thymidine, leaving a single strand gap behind. DNA polymerases attempt to repair this gap by filling it with newly synthesized DNA, reintroducing the mismatched thymidine opposite the O⁶-methylguanine adduct and subsequently requires MMR again. The continuous failing at removing the erroneous pairing will result in a permanent single strand gap that will cause a DSB during the DNA replication in the following S-phase, which induces apoptosis of the cell [55]. As can be concluded from this event, the MMR pathway can be a crucial factor in resistance to methylating agents besides MGMT activity. Indeed, for epigenetic silencing of MGMT to prevent resistance to TMZ of gliomas, it requires the MMR pathway to be functional (Figure 5).

Reversal of MGMT induced resistance

Given that it has been established that MGMT expression is inversely related with resistance to alkylating agents, there has been an increasing focus on discovering possible inhibitors of its activity to reduce and potentially reverse this resistance in tumors [56]. Specifically, the removal of alkyl adducts by MGMT being a stoichiometric reaction has generated interest in researching pseudosubstrate agents that function as inhibitors by depleting MGMT in tumor cells during chemotherapy based on alkylating agents. The most promising of these pseudosubstrate agents so far are O⁶-benzylguanine (O⁶-BG) and O⁶-(4-bromophenyl)guanine (O⁶-BTG, Lomeguatrib). Both MGMT inhibitors have entered phase I and II clinical trials. The safety and toxicity of utilizing O⁶-BG for MGMT depletion

to augment the cytotoxicity of alkylating agents has been evaluated in several phase II clinical trials spanning multiple tumor types and grades (see Table 3). One phase II study of O⁶-BG in combination with TMZ in patients with glioblastoma multiforme and anaplastic glioma resulted in a 3% and 16% response rate, respectively [57]. This combination of O⁶-BG and TMZ was also studied in pediatric patients with recurrent or progressive high-grade gliomas (4% response rate) and brainstem gliomas (0% response rate) [58]. Combining O⁶-BG with BCNU as a treatment for patients with multiple myeloma led to an overall response rate of 27% including 1 complete response and 3 partial responses [59]. In another study this same combined treatment led to no complete or partial responses in 18 patients with nitrosourea-resistant recurrent or progressive malignant glioma [60]. No response was observed in patients with advanced soft tissue sarcoma either after treatment with a combination of O⁶-BG and BCNU [61]. Treatment of patients with advanced melanoma with the combination of O⁶-BG and BCNU resulted in a 5% response rate [62].

Besides O⁶-BG, the only other inactivator of MGMT that has entered clinical trials is O⁶-BTG (see Table 3). It is regarded as the most potent MGMT inactivator with an IC₅₀ (concentration needed to reduce MGMT activity by 50%) of 3.4nM compared to the IC₅₀ of 180nM of O⁶-BG [63]. In one randomized phase II trial, patients with metastatic cutaneous melanoma were treated with either a combination of O⁶-BTG and TMZ (17.3% RR) or TMZ alone (13.5% RR) [64]. Another phase II study in patients with metastatic colorectal carcinoma evaluated the effect of a combination of O⁶-BTG and TMZ or TMZ alone as treatment, both showed no response in all patients [65]. A third phase II study studied the MGMT depletion in patients with metastatic melanoma after treatment with the combination of O⁶-BTG and TMZ or TMZ alone, showing a slow and incomplete inactivation following treatment with TMZ and more rapid, total, depletion with O⁶-BTG and TMZ combined [66].

Up to now, the results of these phase II trials of MGMT inhibitors have been disappointing so far. Although there are many indicators that MGMT is a major resistance factor in many human tumors to multiple chemotherapeutic agents, there has been no convincing evidence so far that the strategy of reversing the resistance by depleting MGMT improves the outcome in cancer patients treated with these agents. The

Table 3. Clinical phase II studies with O⁶-BG and O⁶-BTG.

Drug combination	Cancer type	Results	Reference
Clinical phase II studies with O⁶-BG			
TMZ+O ⁶ -BG	Glioblastoma multiforme	3% RR	[57]
	Anaplastic glioma	16% RR	
TMZ+O ⁶ -BG	Progressive high-grade glioma	4% RR	[58]
	Brainstem glioma	0% RR	
BCNU+O ⁶ -B	Multiple myeloma	27% RR	[59]
BCNU+O ⁶ -BG	Recurrent or progressive glioma	0% RR	[60]
BCNU+O ⁶ -BG	Advanced soft tissue sarcoma	0% RR	[61]
BCNU+O ⁶ -BG	Advanced melanoma	5% RR	[62]
Clinical phase II studies with O⁶-BTG			
TMZ alone	Metastatic cutaneous melanoma	13.5% RR	[64]
TMZ+O ⁶ -BTG		17.3% RR	
TMZ alone	Metastatic colorectal carcinoma	0% RR	[65]
TMZ+O ⁶ -BTG		0% RR	
TMZ alone	Metastatic melanoma	Partial MGMT depletion	[66]
TMZ+O ⁶ -BT		Complete MGMT depletion	

lack of encouraging phase II results in reversing the resistance by the aforementioned treatment combinations might be due to multiple factors. First, dose reductions in TMZ were necessary to avoid hematologic toxicity when combined with O⁶-BG or O⁶-BTG. This could be an underlying factor for its failure to properly sensitize the tumors to the treatment. Alternative methods of administration of either alkylating agents or MGMT inactivators could be a potential solution in reducing system toxicity, enabling the use of higher doses. Second, the dosing regimen of TMZ in combination with an MGMT inactivator is most likely not optimal yet. Thirdly, some of the investigated tumor types did not respond to TMZ alone either and it has not been demonstrated that their resistance is related to MGMT overexpression. Addressing these issues could lead to better results in sensitizing tumors to TMZ and other alkylating agents.

BRCA1 methylation

The breast cancer 1 (BRCA1) gene was first identified in 1994 [37]. It produces the breast cancer type 1 susceptibility protein which is expressed in the cells of breast and other tissues and functions in the BRCA–Fanconi anemia pathway. This protein has an important role in repair of DSB in DNA by forming a large multi-subunit protein complex known as the BRCA1-associated genome surveillance complex (BASC). This large protein complex, also consisting of other tumor suppressors, DNA damage sensors and signal transducers, participates in

the HR mechanism of DNA DSB repair. As mentioned earlier, HR repair uses an undamaged DNA template of either a sister chromatid or homologous chromosome to repair the damaged strands [31]. In nuclear foci in mitotic cells BRCA1 associates and colocalizes with RAD51, a protein involved in the search for homology and strand pairing stages of the HR repair process. This was one of the earliest indications that BRCA1 functions in HR repair [67]. Although the exact mechanisms by which BRCA1 functions in HR are not entirely clear yet, studies have suggested 2 possible roles: It promotes via interaction with CtBP-interacting protein (CtIP) [68], and it might act as a recombination mediator/comediator by interacting with RAD51 [69]. It is generally believed that BRCA1 maintains genomic stability by promoting error free HR and suppressing error prone NHEJ, protecting the DNA from mutagenesis due to error prone nonhomologous DSB repair [70–72]. Indeed, loss of function of BRCA1, whether due to hypermethylation or other means, is a major contributing factor in tumorigenesis.

BRCA1 methylation in cancer

Promoter hypermethylation of the BRCA1 gene has a high frequency of occurrence in both ovarian cancer and sporadic breast cancer. Due to the important role of BRCA1 in HR repair of DNA DSBs, loss of its function has been associated with resistance to platinum-based chemotherapy. These platinum containing drugs belong to the alkylating-like agents as they attach platinum instead of alkyl groups as adducts on DNA. Loss of BRCA1 due to an inactivating mutation has been associated with increased sensitivity to these platinum containing alkylating-like agents in patients with ovarian cancer [73]. As hypermethylation of gene promoter regions silence that particular gene, this should cause loss of function as well, however, research on the methylation status of BRCA1 is sparse. A study has shown that methylation of BRCA1 sensitizes breast cancer cells to PARP inhibitors, another class of drugs used for treating breast cancer patients [74]. Of 167 patients with breast cancer that received adjuvant chemotherapy in the form of PARP inhibitors, patients with BRCA1-methylated tumors had a superior 10-year disease-free survival (78% versus 55%, $P=0.009$) and 10-year disease-specific survival (85% versus 69%, $P=0.024$) than those with BRCA1-unmethylated tumors [75]. BRCA1 hypermethylation has also been associated with a significant increase in median PFS of ovarian cancer patients receiving adjuvant platinum-taxane-based chemotherapy [76]. Another recent study observed that, as is the case with tumors containing BRCA1 inactivating mutations, breast and ovarian cancers containing hypermethylated BRCA1 are more sensitive to platinum-based agents such as cisplatin as well [77]. This suggests that methylation of BRCA1 prevents the HR pathway from successfully repairing the DNA damage induced by platinum-based agents. A study analyzing the DNA methylation status of various genes involved in the HR repair pathway in breast cancer samples, found only hypermethylation of BRCA1 and RNF8 to be associated with chemosensitivity [78].

MLH1 methylation

MLH1 is part of the heterodimeric MLH1-PMS1 protein crucial for creating incisions in DNA during MMR, a repair pathway that corrects replication errors due to the incorporation of the wrong nucleotide (a mismatch), nucleotide deletions and insertions. These incisions are made on either the 3'-or 5'-end of the lesion containing the mismatch after which the ssDNA segment including the mismatch is removed by EXO1, an exonuclease. This enables that the resulting gap can be filled up with newly synthesized DNA that correctly pairs with the already existing DNA strand. As briefly mentioned earlier, the MMR pathway plays an important role in the mechanism by which TMZ attempts to kill cancer cells.

MLH1 methylation in cancer

Methylation of MLH1 is not uncommon in colorectal cancers, as approximately 15% shows micro satellite instability due to methylation of this MMR gene [79]. However, not only does the methylation of MLH1 promote tumorigenesis, it also enables resistance of these tumors to Fluorouracil (5-FU), a widely used chemotherapeutic agent for treating colorectal cancers [80]. Indeed, while promoter hypermethylation of all other discussed DNA repair genes prevent/reverse chemotherapy resistance, hypermethylation of MLH1 promotes resistance to chemotherapeutic agents instead.

Besides conferring resistance to 5-FU, MLH1 promoter methylation also promotes resistance to TMZ due to the role of the MMR pathway in how TMZ kills cancer cells. A study in ovarian cancer cell lines showed that inactivation of MGMT and demethylation of MLH1 combined had a greater sensitizing effect of these cells to TMZ than either MGMT inactivation or MLH1 demethylation alone [81]. In another study, using patient material consisting of resected primary tumors of advanced epithelial ovarian cancer, it was shown that methylation of MLH1 is a major molecular cause of acquired resistance to platinum-based chemotherapy as well [82]. This corresponds with an earlier study that suggested methylation of the MLH1 promoter to be a possible mechanism for cisplatin-resistance in ovarian cancer [83]. MLH1 methylation has recently also been associated with oxaliplatin-resistance in gastric cancer patients [84], although repair of oxaliplatin caused DNA adducts is usually considered to be performed by the ERCC1 complex. The promotion of resistance by MLH1 methylation is due the requirement of MMR to be intact for platinum-based anti-cancer drugs to effectively kill cancer cells. Similar to the methylation adduct added by TMZ, platinum adducts cause misincorporation of a base opposite the adduct during replication. This is recognized by the MMR system that subsequently attempts futile repair. This will generate a signal that triggers apoptosis [85].

Reversal of methylated MLH1 induced resistance

Considering that the resistance to several chemotherapeutic agents is conferred by methylation of the promoter region of

the MLH1 gene, reversal of this resistance should be achievable by demethylating these promoter regions. In general, demethylation of DNA is accomplished by inhibiting the DNMTs that have been discussed earlier. As has been mentioned, these DNMTs are crucial for both establishing the methylation pattern (DNMT3a and DNMT3b) as well as maintaining it through DNA replication (DNMT1). Demethylation of DNA is performed by so called DNA methyltransferase inhibitors (DNMTis) or demethylating agents. The two best studied DNA methyltransferase inhibitors are the azanucleosides azacytidine (5-azacytidine) and decitabine (5-aza-2'-deoxycytidine, DAC). These two DNMTis have been approved for clinical use in treatment of myelodysplastic syndromes and low-blast count AML and have shown improvements in survival of patients [86].

An *in vitro* study showed that resistance to 5-FU can be overcome by re-expression of the MLH1 protein through DAC-induced demethylation in hypermethylated colorectal cancer cell lines [87]. Another study showed that treatment with DAC of ovarian and colon tumor xenografts that contain hypermethylated MLH1 promoters reverses their resistance to varying chemotherapeutic agents, including cisplatin, TMZ and carboplatin [88]. Results of a phase II clinical trial suggest low-dose DAC alters the DNA methylation of genes, restoring sensitivity to carboplatin in platinum-resistant ovarian cancer patients, showing a positive correlation of MLH1 demethylation in tumors with PFS [89]. Although the results are encouraging, one must be cautious. While DNA methylation has a frequent occurrence in cancer cells as it is one of the major mechanisms (the other being mutations) by which genome wide gene expression is altered to facilitate transformation of normal cells into malignant cells, DNMTis are not gene specific and as such can have a genome wide demethylating effects and can lead to negative side-effects.

ERCC1 methylation

Promoter methylation of the ERCC1 gene has attracted relatively little attention compared to the MGMT, BRCA1 and MLH1 gene. ERCC1 is a crucial gene in the NER pathway, which has the ability to repair bulky DNA adducts, including platinum-DNA adducts. The product of the ERCC1 gene interacts with XPF, an endonuclease, to form the heterodimeric ERCC1-XPF protein complex, which is involved in the excision of DNA-adducts by performing incisions at the 5'-end of the lesion.

It had already been reported that cancer cells with a deficient NER pathway have an increased sensitivity to platinum agents [90]. Indeed, considering that ERCC1 is a crucial gene for the NER pathway to function correctly, studies have found its mRNA/protein expression levels to have a predictive value for the resistance of cancer cells to platinum-based chemotherapies (e.g., [91,92]). Therefore it is important to understand which mechanism regulates the ERCC1 expression. Epigenetic inactivation of the ERCC1 gene by promoter hypermethylation could be a potential regulator of its expression. However, very few studies have explicitly investigated the correlation

between ERCC1 promoter hypermethylation and therapy resistances. One study found an association of promoter methylation of ERCC1 with cisplatin sensitivity, using 32 glioma samples [93]. This study also observed that ERCC1 promoter methylation was inversely correlated to mRNA expression. Another study investigated the relation between ERCC1 methylation and radio-sensitivity in glioma cell lines and their results indicated there is strong association of the methylation status of ERCC1 with radio-sensitivity [94]. This study also showed that demethylation of the ERCC1 promoter by using 5-azacytidine resulted in a decrease in radio-sensitivity. Similar to alkylating agents, ionizing radiation also exerts its anti-tumor effect through induction of DNA damage.

DNA repair gene methylation status as biomarker

Since it is increasingly accepted that epigenetic mechanisms such as methylation may be just as important as genetic changes in the development of cancer, DNA methylation biomarkers are slowly included in cancer molecular diagnostics. There are two ways to apply the analysis of DNA methylation in cancer. First, DNA methylation has the potential to be used as a biomarker to confirm the presence or absence of a tumor as in early detection or minimal residual disease detection. Second, an already diagnosed tumor can be analyzed for prognostic or predictive methylation biomarkers. So far DNA methylation biomarkers have mainly garnered attention for potential early detection due to aberrant DNA methylation seemingly occurring in early-stage tumors. However, as the various studies described in this article have shown (Table 4), methylation of the discussed DNA repair genes could be viable biomarkers for prognostic value and for predicting chemotherapeutic responses due to their critical role in acquired chemotherapy-resistance of tumors. This would enable the stratification of patients based on these biomarkers, leading to a more 'personalized' treatment of these patients. Besides potentially lessening patient burden due to better chemotherapy selection, stratification can be a critical component to change the perspective from a negative or neutral outcome to one with a positive outcome by identifying the subset of the population most likely to respond to a novel therapy.

Indeed, due to their critical role in the DNA repair pathways and their association with therapy resistances, the methylation status of these genes generally has a prognostic and predictive significance as well. For example, MGMT promoter methylation status was found to be promising prognostic biomarker in patients with glioblastoma multiforme (GBM) treated with radiation and TMZ, as methylated patients demonstrated a median overall survival (OS) of 24.7 months, whereas unmethylated patients demonstrated a median OS of 16.2 months. Progression free survival (PFS) was also higher among methylated than among unmethylated patients (13.3 months vs. 7.8 months) [95]. This is in agreement with results of another study that showed MGMT methylation was correlated with better survival of treated GBM patients with a hazard ratio (HR)

Table 4. DNA repair gene methylation and therapy resistances in cancers.

DNA repair gene methylation	Effect	Chemotherapeutic agent	Cancer type	Reference
MGMT	Sensitizing	TMZ	Glioma	[50]
			Small cell lung cancer	[54]
		BCNU	Glioma	[49,53]
			Breast cancer	[126]
		CCNU	Glioma	[49]
		Streptozotocin	Glioma	[50]
BRCA1	Sensitizing	PARP inhibitors	Colorectal cancer	[127]
			Breast cancer	[74,75]
		Cisplatin	Breast cancer	[77]
			Ovarian cancer	[77]
			Breast cancer	[77]
MLH1	Resistance promoting	5-FU	Ovarian cancer	[77]
			Ovarian cancer	[77]
		TMZ	Colorectal cancer	[80]
			Ovarian cancer	[81]
			Ovarian cancer	[82,128]
ERCC1	Sensitizing	Cisplatin	Ovarian cancer	[82,89]
			Gastric cancer	[84]
		Radiotherapy	Glioma	[93]
			Glioma	[94]

of 0.61 [96]. Moreover the median OS among GBM patients with methylated MGMT promoter tumors was 19 months compared with 13 months in patients with unmethylated MGMT promoter tumors and the median PFS was 8 months for the methylated MGMT promoter status compared with 6 months for the unmethylated MGMT promoter status [97]. These studies (see Table 5) show great promise in its value as prognostic biomarker, but its use as a predictive biomarker is limited. A major factor is that for a predictive biomarker to be of great value, different treatment options must exist that circumvent or reverse the resistance. For example, clinical trials with possible new treatment options such as O⁶-BG and O⁶-BTG so far showed disappointing results, making the predictive value of MGMT methylation as biomarker of limited use, while its use as a prognostic biomarker would be more sensible. Furthermore, another restriction to the clinical utilization of the methylation status of these DNA repair genes is the lack of a validated, standardized promoter methylation assay. Standardization is mainly obstructed by the lack of consensus on which technique is most manageable in clinical setting and which criteria allow an objective assessment of methylation status. Indeed, while identification of biomarkers that predict tumor response to therapy is a critical step in the

Table 5. Examples of studies showing prognostic use of MGMT methylation in glioblastoma patients.

Cancer type	Status	Median OS (months)	Median PFS (months)	Reference
Glioblastoma	Methylated	24.7	13.3	[95]
multiforme	Unmethylated	16.2	7.8	
Glioblastoma	Methylated	19	8	[97]
multiforme	Unmethylated	13	6	
HR				
Glioblastoma multiforme	Methylated	0.61		[96]

development of treatment regimens tailored to individual patients to produce optimal clinical outcomes, the clinical utility of DNA gene promoter methylation status is currently limited to providing overall prognosis estimations.

Conclusion

As can be concluded, DNA repair genes can be considered an interesting subclass of TSGs. As is the case with other TSGs, they are often epigenetically inactivated by promoter hypermethylation causing genomic instability during carcinogenesis. Epigenetic inactivation of DNA repair genes in cancer has been reported for several DNA repair pathways such as NER, direct repair, MMR and HR repair. However, while methylation of DNA repair genes is generally regarded as a negative event due to this, the same methylation of DNA repair genes sensitizes fully grown tumors to varying chemotherapeutic agents in certain cases. Indeed, methylation of DNA repair genes such as MGMT, BRCA1 and ERCC1 increase the therapeutic sensitivity of varying cancer types, providing a potential therapeutic benefit to patients. The most promising example being the increased sensitivity to the chemotherapeutic agent TMZ due to MGMT promoter hypermethylation in patients with glioma. Surprisingly however, epigenetic inactivation of the MLH1 gene, part of the MMR pathway, has been associated with an increase in resistance of cancer cells to varying chemotherapeutic agents. These findings suggest that the methylation status of these genes could be viable predictive biomarkers and subsequently enable the potential to develop personalized chemotherapeutic regimens tailored to a patient's DNA repair gene methylation status in the future. This however, requires further improvement and advancement in standardization of methylation status assays before clinically applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	JRJ	GJP
Research concept and design	✓	✓
Collection and/or assembly of data	✓	--
Data analysis and interpretation	✓	--
Writing the article	✓	✓
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