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Review

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DNA alkylation lesion repair: outcomes and implications in cancer chemotherapy

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Abstract: Alkylated DNA lesions, induced by both exogenous chemical agents and endogenous metabolites, represent a major form of DNA damage in cells. The repair of alkylation damage is critical in all cells because such damage is cytotoxic and potentially mutagenic. Alkylation chemotherapy is a major therapeutic modality for many tumors, underscoring the importance of the repair pathways in cancer cells. Several different pathways exist for alkylation repair, including base excision and nucleotide excision repair, direct reversal by methyl-guanine methyltransferase (MGMT), and dealkylation by the AlkB homolog (ALKBH) protein family. However, maintaining a proper balance between these pathways is crucial for the favorable response of an organism to alkylating agents. Here, we summarize the progress in the field of DNA alkylation lesion repair and describe the implications for cancer chemotherapy.

Key words: Alkylation repair; Base excision repair; Methyl-guanine methyltransferase (MGMT); AlkB homolog (ALKBH)

1 Introduction

The importance of the inevitable DNA base adduct is dictated by the variable biological effects elicited in cells. Even for one single type of DNA base adduct, the biological effects can vary depending on its chemical structure, distribution within the genome, response pathways or repair mechanisms, and other factors. DNA alkylation, upon which a small or more complex alkyl group is added, constitutes one major class of DNA base adduct in the biological system. Because of its undesirable causes and deleterious effects on genome integrity, DNA alkylation is generally considered a type of DNA lesion (Fu et al., 2012; Soll et al., 2017). To counter alkylated DNA lesion in cells, individual factors are mobilized to recognize each specific adduct, calling for an interplay or competition of multiple highly conserved repair pathways. Consequently, the conversation between these repair pathways could either sequester or further elicit genotoxicity, therefore complicating the biological effects in cells (Tsuzuki et al., 1998; Fu et al., 2012; Soll et al., 2017). Meanwhile, researchers are systematically considering alkylating agents that induce alkylation lesions in cells as a promising chemotherapeutic option for cancer treatment. Because of the agents' broad activity, each alkylating agent can induce a spectrum of alkylation lesions in cells, in turn eliciting convergence of different repair pathways and further complicating the biological effects (Drabløs et al., 2004; Fu et al., 2012; Soll et al., 2017). In this review, we focus on the regulation and the outcomes of the DNA alkylation lesion repair, and how these outcomes influence carcinogenesis and individual response to cancer chemotherapy.

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2 Complexity in DNA alkylation lesions

DNA alkylation lesions generated in the biological system are unavoidable due to the ubiquitously presented alkylating agents and the high nucleophilic

character of DNA bases. Each alkylating agent has broad reactivity and may induce numerous alkylation lesions. These lesions vary in terms of their chemical structure and distribution in the genome, leading to variable consequences in cells.

2.1 Alkylating agents

Alkylating agents are basically chemical compounds which can reactively transfer alkyl carbon groups onto the high nucleophilic nitrogen or oxygen atoms on DNA bases (Shrivastav et al., 2010; Fu et al., 2012; Soll et al., 2017). These compounds may arise both endogenously and exogenously. While external sources are well documented, the internal donor of the alkyl group is not clearly defined. It has been suggested that the methyl donor *S*-adenosylmethionine (SAM), along with several other methyl metabolites, may serve as a potential source (Barrows and Magee, 1982; Rydberg and Lindahl, 1982). There are five major classes of alkylating agents, differing in the number of reactive sites, the type of alkyl groups transferred, or the particular chemical reactivity (Table 1). Usually, a bifunctional alkylating agent contains more than one reactive site, which can react with separate DNA bases to form cross-links or bulky DNA adducts. Conversely, a monofunctional alkylating agent contains

Table 1 Alkylating agents and induced lesions

Category	Name	Mono (M)/Bi (B) functional	S_N1/S_N2	DNA lesion	Clinical application
Alkyl sulfonates	Busulfan	B	S_N2	G-A cross-link G-G cross-link	Leukemia, polycythemia vera, myeloid metaplasia
	Methyl methanesulfonate	M	S_N2	N^7meG N^3meA N^1meA N^3meC	N/A
Triazene	Dacarbazine	M	S_N1	N^7meG N^3meA O^6meG	Metastatic melanoma, Hodgkin's lymphoma, sarcoma
	Procarbazine				Malignant glioma, Hodgkin's lymphoma
	Temozolomide				Malignant glioma
	Streptozotocin				Pancreatic islet cell cancer
Nitrosoureas	Nimutine	M	S_N1	N^7-alkylG O^6Cl-ethylG	Brain tumors, solid tumors
	Carmustine			N^1,O^6-EG G-C cross-link	Brain tumors, lymphoma, melanoma
	Lomustine			G-G cross-link	Brain tumors, lymphoma, melanoma
	Fotemustine				Metastatic melanoma
Nitrogen mustards	Mechlorethamine	B	S_N1	N^7-alkylG O^6-alkylG G-G cross-link G-A cross-link	Lymphoma, leukemia, multiple myeloma, ovarian cancer, solid tumors
	Cyclophosphamide				
	Melphalan				
	Bendamustine				
	Chlorambucil				
Aziridines	Altretamine	B	S_N1	N^7-alkylG O^6-alkylG G-G cross-link G-A cross-link	Recurrent ovarian cancer
	Mitomycin C				Breast cancer, gastrointestinal tumor
	Thiotepa				Breast cancer, ovarian cancer, bladder cancer

S_N1: monomolecular nucleophilic substitution; S_N2: bimolecular nucleophilic substitution 2; N^7meG: N^7-methylguanine; N^3meA: N^3-methyladenine; N^1meA: N^1-methyladenine; N^3meC: N^3-methylcytosine; O^6meG: O^6-methylguanine; O^6Cl-ethylG: O^6-chloroethylguanie; N^1,O^6-EG: N^1,O^6-ethanoguanine.

only one reactive group, either a simple methyl or a complex alkyl group. All of the alkylating agents attack DNA bases through a nucleophilic substitution reaction (monomolecular nucleophilic substitution (S_N1) or bimolecular nucleophilic substitution 2 (S_N2)). S_N2-type alkylating agents mainly react with the ring nitrogen atoms on the bases, while S_N1-type alkylating agents can react with both nitrogen and excircle oxygen atoms on the bases. Notably, most clinics favor S_N1-type alkylating agents (Table 1), whereas S_N2-type alkylating agents may also be beneficial due to their relative selectivity.

2.2 Variety of alkylation lesions induced in cells

Depending on the type of alkyl groups and the position of the bases during the reaction, there are a wide variety of alkylation lesions, ranging from large helix distortions to small base adducts (Table 1). For example, the chloroethyl group provided by chloroethylating agents such as nitrosourea compounds can react with the N^7 and O^6 positions of guanine to generate chloroethyl adducts. The O^6 chloroethyl guanine may react with the nearby cytosine through a spontaneous chemical rearrangement, generating a guanine-cytosine intrastrand or interstrand cross-link (Warren et al., 2006). These lesions are highly cytotoxic and require the involvement of the nucleotide excision repair (NER) or the Fanconi anemia (FA) pathway (Hong et al., 2007; Deans and West, 2011).

Methyl DNA adducts, introduced by most clinically used methyl alkylating agents, are the simplest alkylation lesions and cause less distortion in the DNA. Because of the high nucleophilic character, nearly 70% of the total alkylation lesions in cells, induced by either S_N1 or S_N2 monofunctional alkylating agents, are the relatively innocuous N^7-methylguanine type (N^7meG). However, N^7meG spontaneously depurates to generate an abasic (AP) site, which may cause replication collapse and have a tendency to introduce mutations (Beranek, 1990; Gentil et al., 1992). The less popular N^3-methyladenine type (N^3meA) (about 10%) causes more replication block but less transcription block, because it usually presents in the minor groove of DNA helices, where most error-free DNA polymerases, but not RNA polymerase II, are loaded (Morales and Kool, 1999; Naryshkin et al., 2000). Meanwhile, translesion polymerases can bypass such lesions, but at the risk of more mutations (Larson et al., 1985; Johnson et al., 2007). *O*-methyl lesions, including O^6-methylguanine (O^6meG) and O^4-methylthymine (O^4meT), are potentially mutagenic because of the readily formed mispairs, which can also be cytotoxic in cells through the subsequent processing of the mismatch repair (MMR) machinery (Warren et al., 2006). N^1-methyladenine (N^1meA) and N^3-methylcytosine (N^3meC) lesions, minor lesions mainly induced by S_N2 alkylating agents, disrupt the base pair and are intrinsically cytotoxic, but less likely to be mutagenic (Shrivastav et al., 2010). The diversity of the types of DNA alkylation damage necessitates the involvement of a number of DNA repair systems, which include multi-step base-excision repair (BER), direct reversal by methylguanine methyltransferase (MGMT), and direct demethylation by the AlkB homolog (ALKBH) family proteins (Fig. 1). These mechanisms collectively modulate the biological effects of alkylation lesions and in turn provide strong instructions for cancer prevention or treatment.

3 BER as the major tool for alkylated DNA lesion

In either prokaryotic or eukaryotic system, BER is the primary mechanism for removing small DNA base lesions, including most of the alkylated lesions. BER occurs through two major sub-pathways which differ in the repair gap size: short-patch or long-patch BER. Both sub-pathways share general procedures including the recognition and removal of the damaged nucleotide, gap filling by DNA synthesis, and subsequent ligation into the pre-existing DNA strand (Fig. 1a). The multistep nature of the BER pathway inevitably gives rise to several toxic intermediates (5'-deoxyribose phosphate (5'-dRP), AP site, single-strand break (SSB)), thus necessitating proper coordination between BER pathway players.

3.1 Recognition of alkylated lesions by DNA glycosylase

To initiate the BER pathway in cells, a specific DNA glycosylase is usually required to correctly recognize and excise the damaged base. The substrate preference of a glycosylase determines its role in particular repair pathways (Svilar et al., 2011). In dealing with alkylated lesions, the predominant glycosylase in

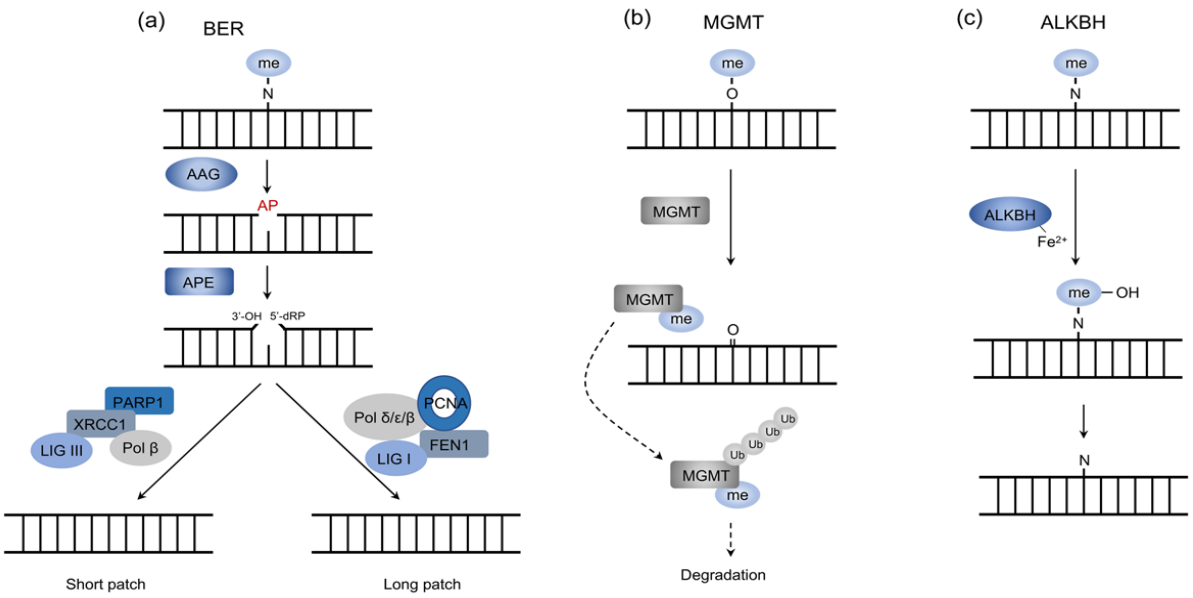

Fig. 1 Essential repair mechanisms for alkylation lesions. (a) Repair of an *N*-alkyl DNA lesion by multi-step base excision repair (BER). In cells, BER can occur in two sub-pathways: short-patch or long-patch BER. In both sub-pathways, the *N*-methyl base adduct is first removed by alkyladenine-DNA glycosylase (AAG) to generate an abasic (AP) site. Then, the AP site is cleaved by the AP endonuclease (APE) to generate a single-strand break (SSB) with flapped 5'-deoxyribose phosphate (5'-dRP) or flapped 3'-hydroxyl (3'-OH). In short-patch BER, DNA polymerase β (Pol β) can remove the 5'-dRP moiety and fill in the gap; then ligase III (LIG III) ligates the gap into the existing DNA. This process is coordinated by the scaffold activity of X-ray repair cross-complementing group 1 (XRCC1). Additionally, poly(ADP-ribose) polymerase 1 (PARP1) may help to recruit the XRCC complex. In long-patch BER, DNA polymerase δ/ε (Pol δ/ε) recruited by proliferating cell nuclear antigen (PCNA) synthesizes DNA to fill in the gap, leaving a long stretch of nascent DNA. Then, flap endonuclease 1 (FEN1) removes stretch from the bases. Finally, ligase I (LIG I) ligates the gap and the repair is finished. (b) Direct reversal of an *O*-alkyl DNA lesion by O^6-methylguanine-DNA methyltransferase (MGMT). The methyl group on the *O*-methyl base adduct is directly transferred to a catalytic residue of MGMT. Next, MGMT is ubiquitinated and subject to proteasome degradation. (c) Direct demethylation of an *N*-alkyl lesion by AlkB homolog (ALKBH) demethylase.

higher eukaryotic cells is the alkyladenine-DNA glycosylase (AAG; also known as *N*-methylpurine DNA glycosylase (MPG)). AAG exclusively catalyzes the hydrolysis of the *N*-glycosidic bond between the alkylated base and the ribose, thereby generating an AP site for subsequent repair. Both in vitro assays and genetic studies have confirmed that most of the alkylated purines (e. g., N^7meG, N^3meA) are preferentially repaired by AAG (Lindahl, 1993; Jacobs and Schär, 2012). However, $Aag^{-/-}$ mice exhibited neither significant development defects nor severe spontaneous mutation rates (Engelward et al., 1997), indicating that these alkylated lesions induced by endogenous alkylating agents can be tolerated by other back-up repair mechanisms (such as translesion synthesis (TLS) or MMR). Conversely, it is believed that the excessive activity of AAG may introduce more AP sites in cells, which are more detrimental. Importantly, compared to normal littermates, $Aag^{-/-}$mice are more prone to develop colon cancer induced by azoxymethane-dextran sodium sulfate (AOM-DSS) (Meira et al., 2008). This suggests that AAG mainly protects against mutagenicity rather than the cytotoxicity elicited by overwhelming alkylation lesions.

While AAG spontaneously and effectively searches its substrate in the native DNA context, substrate searching in the chromatin is extremely inefficient (about 80% reduction), as revealed by in vitro assay (Olmon and Delaney, 2017). This raises the question of how AAG effectively recognizes the alkylated purines in cells. There are two major models recently proposed. (1) Recruitment of AAG on lesions by transcription or replication machinery, as supported by a relative predisposition of BER to occur on the active transcribing or replicating regions (Odell et al., 2013; Bjørås et al., 2017). Biochemical studies showed that AAG interacts with both transcription repressors (e.g., methyl-CpG-binding domain protein 1 (MBD1)) and elongators (e.g.,

elongator complex protein 1 (ELP1)) (Watanabe et al., 2003; Huttlin et al., 2017; Montaldo et al., 2019). These interactions enhance the AAG-mediated repair along transcription units and may also contribute to localized gene expression (Montaldo et al., 2019). However, a thoroughly developed genome-wide profiling approach is needed to dissect the distribution of AAG and its associated chaperons in the genome. (2) Reorganization of the chromatin structure to favor the repair, as supported by the different chromatin structures observed before and after BER (Odell et al., 2013). Importantly, these favorable chromatin structures are documented to be pivotal for most of the downstream factors in BER, including AP endonuclease 1 (APE1), X-ray repair cross-complementing group 1 (XRCC1), and polymerase β (Pol β) (Odell et al., 2011; Rodriguez and Smerdon, 2013; Rodriguez et al., 2017). However, firm evidence demonstrating that AAG associates with any chromatin structures or histone marks is lacking. In any case, the difference in the BER factor loading demonstrates an inequality of repair across the genome. Whether this inequality in cells contributes to tissue or organ specificity is another interesting question which may widen our understanding of the biological effects of alkylation lesions.

3.2 Deleterious consequences of BER imbalance

After initiation by DNA glycosylases, imbalance in each step of BER may cause accumulation of toxic intermediates and have a profound biological effect (Fig. 2a). These intermediates include the genotoxic AP site, 5'-dRP or 3'-hydroxyl (3'-OH), and SSB (as generated sequentially by AAG, APE1, and Pol β) (Fig. 1a). Unlike the original alkylated lesions, these unrepaired intermediates can consequently collapse to double-strand breaks (DSBs), which may introduce more severe deleterious effects due to excessive activity of homologous recombination (HR) (Sossou et al., 2005; Ströbel et al., 2017; Yuan et al., 2017). Indeed, excessive activity of AAG or loss of the APE1 in cells causes higher microsatellite instability, spontaneous frameshift mutagenesis, and chromatin aberrations (Coquerelle et al., 1995; Hofseth et al., 2003; Klapacz et al., 2010). Another key BER protein, XRCC1, acts as a scaffold to coordinate the DNA excision and ligation by interacting with both DNA Pol β and ligase III (LIG III) (Fig. 1a). Hence, cells deficient in *XRCC1* display accumulated SSB and severe hypersensitivity to alkylating agents (Fan et al., 2007; Hoch et al., 2017). Moreover, polymorphisms in *XRCC1* are found to correlate with the development of several solid tumors (Jiang et al., 2009). Similarly, alterations in *Polβ* are observed in almost 30% of human cancers (Starcevic et al., 2004). Apart from DNA polymerase activity, Pol β also excises the 5'-dRP moiety (Feng et al., 1998). Mutagenesis study has demonstrated that missense mutations which disrupt the excision activity of Pol β confer hypersensitivity to alkylating agents (Sobol et al., 2000), suggesting high cytotoxicity of the 5'-dRP moiety. Collectively, the imbalance of BER conferred by abnormal alteration of BER players poses a huge threat to genome integrity; it can significantly influence carcinogenesis and alkylation chemotherapy response.

3.3 Competing mechanisms for processing intermediates

The presentation of intermediates in the BER pathway may also engage the convergence and competition of the BER sub-pathways. Though it remains unclear how the sub-pathway transition occurs, it is certain that the initiation by the specific glycosylase, the size of the repaired gap, and the cell cycle are all critical. At the molecular level, the processing of the 5'-dRP moiety may well be the turning point (Fig. 1a). In short-patch BER, the 5'-dRP moiety is removed by Pol β and subsequently filled, with the XRCC1 working as a scaffold. In long-patch BER, these gaps are branched by proliferating cell nuclear antigen (PCNA); therefore, the replicative DNA polymerase δ/ε (Pol δ/ε) can fill the gap. Thus, long-patch BER may largely associates with the replication machinery and mainly occurs during replication. However, it is not clear whether long-patch BER can also occur in non-proliferating cells. One model to explain the molecular mechanism concerning the sub-pathway choice suggests that high-mobility group box 1 (HMGB1) acts as an important director toward long-patch BER. Mechanistically, HMGB1 can block the 5'-dRP moiety from Pol β and at the same time interact with enzymes which play a role in the long-patch BER pathway (Prasad et al., 2007; Liu et al., 2010). However, it is unclear whether HMGB1 acts exclusively as the sole switcher, as it also functions in multiple DNA repair pathways. In addition, 5'-dRP lesions could be initially processed by Pol β and then

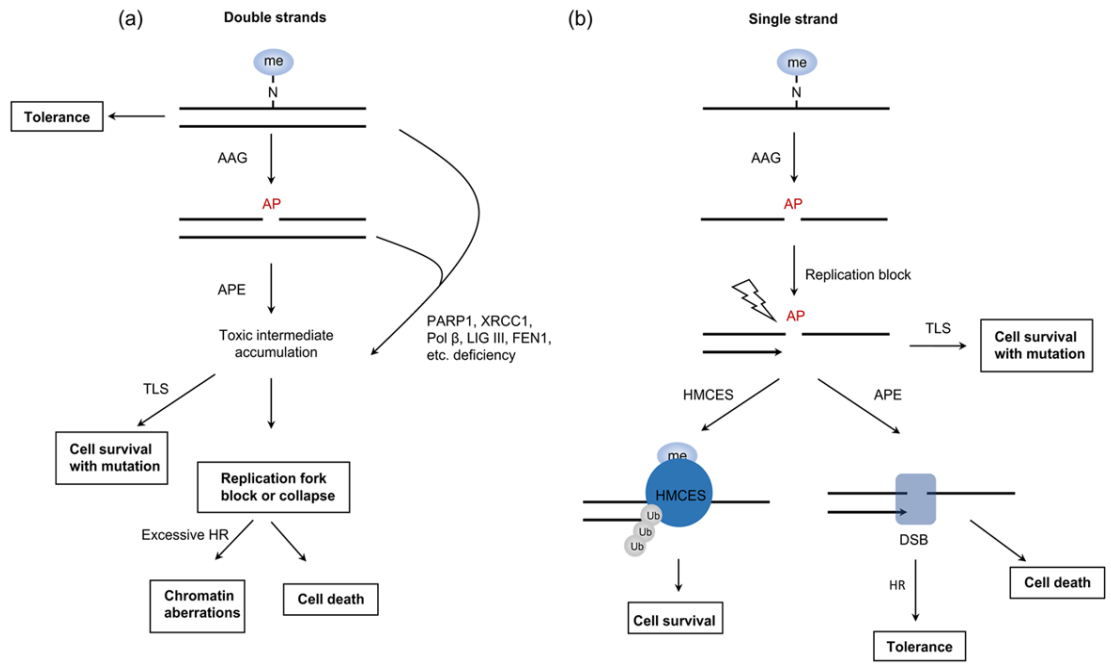

Fig. 2 Biological effects of alkylated purine repair. (a) Consequences of *N*-alkyl lesion repair by base excision repair (BER) within a double-stranded DNA (dsDNA) context. Several types of original *N*-alkyl lesions are not inherently toxic and can be tolerated by cells. Removal of *N*-methyl DNA base adducts by alkyladenine-DNA glycosylase (AAG) generates toxic abasic (AP) sites. High AAG activity in cells leads to accumulation of AP sites, which will lead to replication fork block or collapse, and ultimately to cell death. However, the translesion synthesis (TLS) mechanism can bypass AP sites to protect cells from death, but with more mutations. Defects in BER efficiency caused by deficiency in certain BER factors (AP endonuclease 1 (APE1), poly(ADP-ribose) polymerase 1 (PARP1), X-ray repair cross-complementing group 1 (XRCC1), polymerase β (Pol β), ligase III (LIG III), flap endonuclease 1 (FEN1), etc.) will cause accumulation of toxic intermediates, which also leads to replication fork block or collapse, followed by cell death. (b) Consequences of *N*-alkyl lesion repair within a single-stranded DNA (ssDNA) context. When an *N*-methyl DNA base adduct presents in ssDNA, it is first recognized and removed by AAG to generate AP sites, which blocks replication. TLS can bypass such blocks to protect cells, but with more mutations. APE-mediated AP site resection leads to the generation of double-strand breaks (DSBs). The homologous recombination (HR) mechanism can tolerate the cytotoxicity of DSBs, otherwise this would lead to cell death. Alternatively, the AP site generated in the ssDNA can be protected by formation of a 5-hydroxymethylcytosine (5hmC) binding, ES-cell-specific (HMCES)-DNA complex, which will eventually be resolved by proteasome degradation.

subject to Pol δ/ε, either in a PCNA-dependent or -independent manner (Asagoshi et al., 2010). Nevertheless, these two sub-pathways occur simultaneously in most cells, with long-patch BER exhibiting higher efficiency in repairing longer gaps than short-patch BER.

Another competition may occur in the processing of AP sites generated by AAG. Recent studies showed that loss of a novel AP site sensor 5-hydroxymethylcytosine (5hmC) binding, ES cell-specific (HMCES) in cells causes accumulation of AP sites, either spontaneously or in response to methyl-methane sulfonate (MMS), suggesting a role for HMCES in relieving the cytotoxicity of AP sites (Mohni et al., 2019; Mehta et al., 2020). In vitro assay indicates that HMCES covalently attaches to AP sites in single-stranded DNA (ssDNA) to form a DNA protein complex (DPC), which protects genome stability in S phase (Mohni et al., 2019). Interestingly, lack of *HMCES* not only sensitizes cells to alkylating agents, but also induces higher mutagenicity, suggesting that factors in these mechanisms may compete with each other (Fig. 2b) (Mohni et al., 2019). Unfortunately, the detailed repair mechanism of HMCES-mediated repair is not well defined. The clinical relevance of *HMCES* and alkylating chemotherapy drug response needs further investigation.

3.4 BER pathway and implications for cancer chemotherapy

BER players have been documented as important modulators in cancer chemotherapy. For example,

APE1 endonuclease activity on AP sites contributes to the alkylating agent resistance in medulloblastomas and neuroectodermal tumors (Bobola et al., 2005; Dumitrache et al., 2018). Mouse embryonic fibroblasts (MEFs) deficient in *Polβ* are hypersensitive to alkylating agents (Horton et al., 2003; Poltoratsky et al., 2005). Interestingly, MMS-induced alkylation damage is tolerated in *Polβ*-deficient MEF cells after subsequent loss of AAG (Horton et al., 2003; Poltoratsky et al., 2005), supporting the importance of proper coordination between BER factors.

On the other hand, developing BER inhibitors seems promising for cancer treatment. For example, methoxyamine, one well known clinically used APE inhibitor, has been successful in numerous cancer cell types and tumor xenograft models in combination with alkylating agents (Taverna et al., 2001; Liu and Gerson, 2004; Bapat et al., 2010). Mechanistically, methoxyamine reacts with an aldehyde-sugar group of AP site and forms a stable methoxyamine-AP-intermediate, which blocks the endonuclease activity of APE and disrupts the balance of BER (Taverna et al., 2001; Bapat et al., 2010). Similarly, inhibition of Pol β also unbalances BER and shows promising effects in alkylation chemotherapy. Clinical trial data have suggested that inhibitors of Pol β are effective in combination with temozolomide (TMZ) in colon cancer (Jaiswal et al., 2009, 2011). However, both APE1 and Pol β are essential proteins, as loss of either one in mice is embryonic lethal (Sobol et al., 1996; Bapat et al., 2009). Therefore, administration of these inhibitors may also be detrimental to normal cells. To reach a better therapeutic end, poly(ADP-ribose) polymerase (PARP), loss of which is not lethal in mouse models, has emerged as another intriguing target. Although it is still controversial whether PARP participates in the main stage of BER, it has an important role in the proper repair of SSBs and may contribute to the recruitment of XRCC1 complex. Consequently, PARP1 deficiency in both mice and cancer cells causes increased sensitivity to various alkylating agents (de Murcia et al., 1997; Shibata et al., 2005). Importantly, PARP inhibitors selectively kill cancer cells carrying *BRCA* mutations or with any other specific alterations (Rouleau et al., 2010). This synthetic lethality effect further helps to dissimilate the drug effects in different tissues and different patients. Notably, alteration in the efficiency of BER may render cancer cells resistant to alkylating agents when combined with PARP inhibitors (Jelezcova et al., 2010). This seemingly controversial result comes from a break in the driving chain of the toxic intermediates generated during BER. Collectively, these findings help to establish BER modulations as an essential chemotherapeutic option, while also supporting the need for a comprehensive genetic inspection before clinical treatment.

4 MGMT directs the proper repair of *O*-alkyl lesions

Of all the single-methyl lesions induced by commonly used S_N1-type alkylating agents, *O*-alkyl lesions contribute most to mutagenic and cytotoxic effects in cells. These lesions can be preferentially eliminated through direct reversal by MGMT, which counters the genotoxicity elicited by the intact MMR machinery in cells (Fig. 3). Together, the leverage between the MGMT expression status and the MMR efficiency drives the carcinogenesis potential of *O*-alkyl lesions, as well as regulates the response to most of the S_N1-type alkylating agents.

4.1 Direct reversal of O^6meG by MGMT

MGMT reactively transfers the alkyl groups on DNA bases to a cysteine residue in its catalytic pocket. This process is irreversible because it will inactivate the MGMT, which is then subject to degradation (Fig. 1b) (Kaina et al., 2007). MGMT targets most of the *O*-alkyl lesions in cells, with higher preference to *O*-methyl lesions (such as O^6meG). Other *O*-alkyl lesions, such as the O^6-ethylation of guanine, cause larger distortion of DNA and can also be repaired by the NER or FA pathway. Importantly, there is no significant difference in the repair of O^6meG in either ssDNA or double-stranded DNA (dsDNA), nor any bias toward the active transcription regions or inactive regions. MGMT is more of a protective but not an essential protein, as knockout of MGMT in mice confers no significant abnormality but shows increased toxicity and a high predisposition to tumors upon treatment with exogenous alkylators (Glassner et al., 1999). The protecting effects of MGMT are clearly manifested by alkylation chemotherapy resistance in brain, skin, thymus, and liver cancers with high expression of MGMT (Christmann et al., 2011).

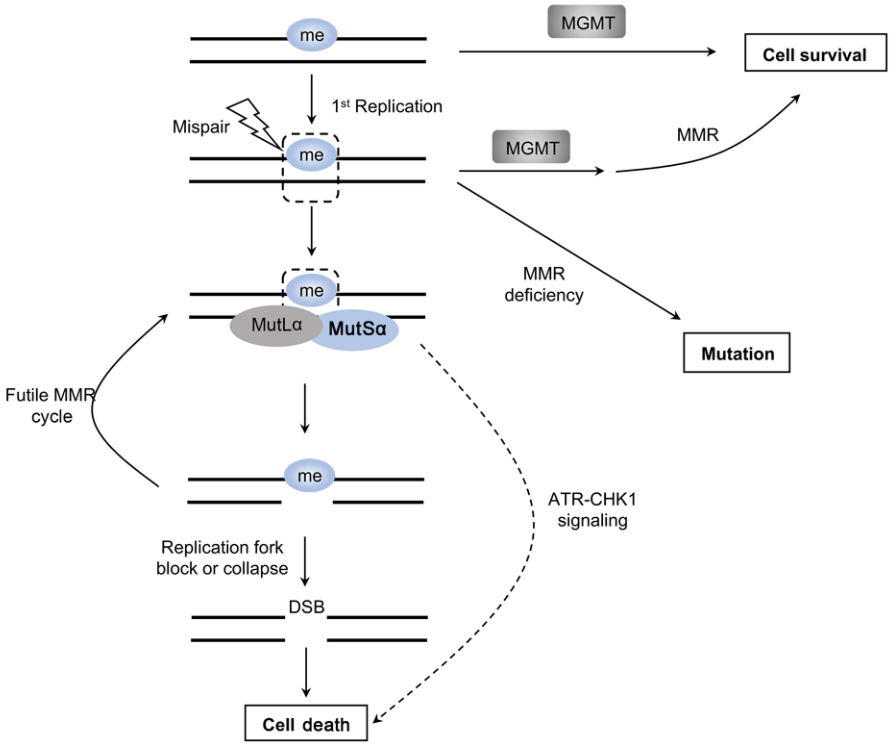

Fig. 3 Biological effects of *O*-alkyl lesion repair. The *O*-alkyl lesion can be directly repaired by *O*^{6}-methylguanine-DNA methyltransferase (MGMT). If the lesion is not properly repaired, DNA replication at the lesion site will introduce mispairs, which can be recognized by the MutSα-MutLα complex and activate mismatch repair (MMR) signaling. The futile cycles of DNA resection and resynthesis can cause replication fork collapse and double-strand break (DSB) formation, which can ultimately lead to cell death. Meanwhile, the mispair recognized by the MutSα-MutLα complex can directly activate ataxia telangiectasia-mutated and Rad3-related (ATR) kinase and induce the ATR-CHK1 (checkpoint kinase 1) checkpoint, which can also contribute to cell death.

4.2 Modulation of the biological effects of *O*^{6}meG by MGMT and MMR

O^{6}meG lesions are potentially mutagenic and carcinogenic, because they cause significant increases in the G:C to A:T transition ratio during DNA replication (Warren et al., 2006). Therefore, the overexpression of MGMT has been shown to prevent lymphoma carcinogenesis, even in a cancer-prone mouse model (Dumenco et al., 1993; Liu et al., 1994). Yet the *O*^{6}meG is inherently less cytotoxic; genetic studies substantiated that efficient MMR machinery is required to elicit its cytotoxicity (Fig. 3). One model explained that the futile cycles of MMR, in which a mispaired nucleotide against an *O*^{6}meG lesion is excised and then resynthesized, eventually lead to replication collapse and trigger the apoptotic response (York and Modrich, 2006; Mojas et al., 2007; Quiros et al., 2010). Importantly, as an iterative round of MMR requires multiple replication processes, *O*^{6}meG shows high cytotoxicity specifically in proliferating cancer cells (Roos et al., 2004). However, genetic studies showed that the missense mutations in mutator S homolog 2 (*Msh2*) or *Msh6*, which have normal affinity to the mispair but defect in ATP processing, still confer a normal apoptotic response to several S_N1 alkylating agents (Lin et al., 2004; Yang et al., 2004). Hence, another model is proposed where the interaction between the MutSα-MutLα complex and ataxia telangiectasia-mutated and Rad3-related (ATR) can directly activate the ATR-ATRIP (ATR-interacting protein) pathway and induce the checkpoint kinase 1 (CHK1) checkpoint; but to what extent these activations alone trigger cell death is currently unclear (Yoshioka et al., 2006). Perhaps both models work together in cells to elicit cytotoxicity in *O*^{6}meG: while direct signaling initiates the immediate apoptotic responses, the futile cycles of MMR amplify and sustain the cytotoxicity.

4.3 Guidance for S_N1 alkylation chemotherapy by the MGMT expression status

In clinic, the expression status of MGMT is an important indicator for diagnosis in several tumors, including glioma and glioblastoma. Determining the epigenetic modification status of the *MGMT* gene could help to improve the efficacy of clinically used alkylating drugs. For example, the front-line chemotherapeutic drug TMZ has been shown effective in glioma and glioblastoma patients with hypermethylation of the *MGMT* promoter region (Esteller et al., 2000; Hegi et al., 2005; Christmann et al., 2011; Butler et al., 2020). In addition, several MGMT inhibitors are utilized in combination with alkylation chemotherapy, such as O^6-benzylguanine (O^6BG), which works by competition with the cellular O^6meG. Patients with anaplastic glioma demonstrate the effectiveness of O^6BG in sensitizing cancer cells to TMZ (Sun et al., 2018; Saha et al., 2020).

In addition, MMR efficiency, which accounts for the cytotoxicity of O^6meG lesion in cells, is another important consideration when exploiting MGMT-dependent chemotherapy. MMR blocking in *Mgmt*$^{-/-}$ mice reduces the sensitivity toward alkylating agents (Kawate et al., 2000; Klapacz et al., 2009). In this manner, loss of *Msh6* or mutator L homolog 1 (*Mlh1*) expression by either epigenetic inactivation or mutation may provide instruction for the proper use of chemotherapeutic alkylators, including nitrosourea and TMZ (Fukushima et al., 2005; Hunter et al., 2006; Xie et al., 2016). Interestingly, whereas loss of MMR efficiency in many colon and gastric cancers is correlated with poor prognosis (McFaline-Figueroa et al., 2015), the neoantigen stem from the increased mutation may guide selective response to immune checkpoint blockage (Westdorp et al., 2016; Germano et al., 2017).

5 Direct reversal of alkylation lesions by the ALKBH demethylase

Another direct reversal mechanism to counter alkylation lesions is the reversal of several cytotoxic *N*-alkyl lesions (such as N^1meA or N^3meC) by the ALKBH demethylase family, in a Fe(II) and 2-oxogluterate (2-OG)-dependent reaction (Fig. 1c). To date, nine human ALKBH homologues are known, but only ALKBH2 and ALKBH3 show strong in vitro activity toward alkylated DNA adducts (Duncan et al., 2002; Aas et al., 2003). Moreover, spontaneous genomic mutations and N^1meA lesions accumulated in *Alkbh2*$^{-/-}$ mice but not in *Alkbh3*$^{-/-}$ mice, suggesting a dominant role for ALKBH2 in reversal of N^1meA lesions (Ringvoll et al., 2006). Further studies found that compared to *Alkbh2*$^{-/-}$ mice, *Alkbh2*$^{-/-}$*Alkbh3*$^{-/-}$ double-knockout mice are more susceptible to alkylating agents, suggesting that both ALKBH2 and ALKBH3 are required for proper alkylation repair in vivo (Calvo et al., 2012). A major difference between these two enzymes is the substrate preference. While ALKBH2 prefers a dsDNA context, ALKBH3 prefers an ssDNA substrate (Aas et al., 2003). Interestingly, the ALKBH3-associated helicase activating signal co-integrator complex 3 (ASCC3) unwinds dsDNA to expose the alkylation lesion, expanding the substrate repertoire for ALKBH3 (Dango et al., 2011).

5.1 Signal transduction in ALKBH-dependent alkylation repair

Repair factor recruitment is essential for proper repair processes. Normally, the substrates for ALKBH proteins are hidden within the DNA double helix, underscoring the importance of a proper recognition mechanism. A possible model for ALKBH2 recruitment has been proposed based on the interaction between PCNA and ALKBH2 through a non-canonical binding motif (Gilljam et al., 2009). Although the inducible interaction and colocalization between PCNA and ALKBH2 in S phase support this theory, knowledge of the detailed coordination and recognition mechanism is still lacking (Gilljam et al., 2009; Fu et al., 2015). Consistent with the ssDNA preference, the recruitment of ALKBH3 may largely depend on the transcription or splicing machinery and the assembly of an ASCC (Fig. 4a). Evidently, deficiency in either one of the above factors impairs ALKBH3 foci formation and sensitizes cells to alkylating agents (Dango et al., 2011; Brickner et al., 2017, 2019; Soll et al., 2018). Additionally, the defects of ALKBH2 foci formation in ASCC2 depletion cells also indicate an overlap in the regulation of ALKBH2 and ALKBH3 recruitment. Moreover, the absence of a ubiquitin-binding domain in ASCC2 diminishes the recruitment of ASCC complex and ALKBH3 to the lesion, suggesting ubiquitin-dependent regulation (Brickner et al., 2017). A subsequent proteomic study identified really interesting new gene finger protein 113A (RNF113A)

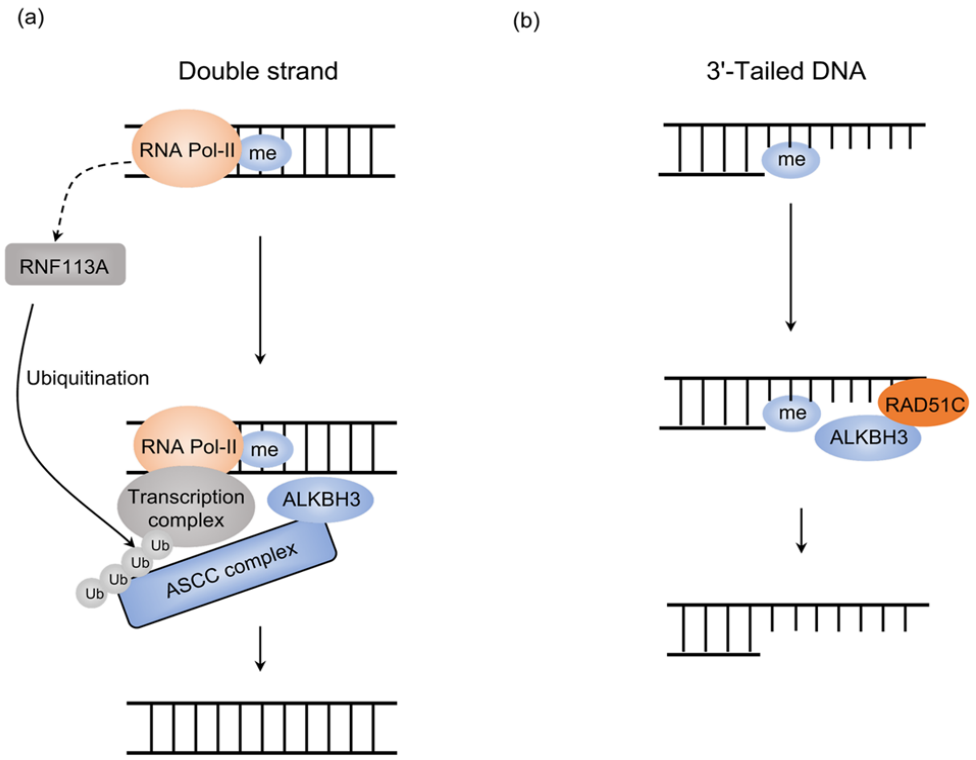

Fig. 4 AlkB homolog 3 (ALKBH3)-dependent repair pathway. (a) Recruitment of ALKBH3 by transcription machinery. When the alkylation lesion is buried in double-stranded DNA (dsDNA), it may require transcription machinery to recognize and initiate the repair. RNA polymerase II (RNA Pol-II) will pause at the alkylated lesion, allowing really interesting new gene finger protein 113A (RNF113A) to ubiquitinate several proteins in the transcription complex. This ubiquitination chain can subsequently recruit the activating signal co-integrator complex (ASCC) complex. Finally, the ASCC complex unwinds the DNA and guides ALKBH3 to repair the lesion. (b) Recruitment of ALKBH3 to 3'-tailed DNA. If the alkylation lesion presents adjacent to double-strand break (DSB), it will first allow the end resection to generate the 3'-tailed DNA. Subsequently, DNA repair protein RAD51 homolog C (RAD51C) binds the 3'-tailed DNA and guides ALKBH3 to repair the lesion.

as the major E3 responsible for the generation of the ubiquitin chain recognized by ASCC2 (Brickner et al., 2017) (Fig. 4). Significantly, nonsense mutation in RNF113A has been found to correlate with X-linked trichothiodystrophy (X-TTD) in *Caenorhabditis elegans* (Corbett et al., 2015). Cells from X-TTD human patients show diminished ASCC recruitment and lower cell viability in response to MMS, indicating a link between X-TTD and ALKBH3-dependent alkylation repair (Brickner et al., 2017). It is questionable whether ALKBH3 is linked with replication where ssDNA is also available, since ALKBH2 but not ALKBH3 interacts with PCNA on replication forks. Interestingly, studies in *Escherichia coli*, yeast, and human cells showed that ssDNA-binding proteins recombinase A (RecA) and DNA repair protein RAD51 homolog C (RAD51C) interact with ALKBH3, thus facilitating the recruitment of ALKBH3 at the 3'-tailed DNA generated during HR repair (Mohan et al., 2019) (Fig. 4b). However, it remains unclear whether a similar mechanism may also guide ALKBH3 demethylase alkylation lesions at replication forks.

5.2 ALKBH-dependent alkylation repair and cancer chemotherapy

Deficiency in ALKBH3 or ASCC3 increases cellular sensitivity to alkylating agents, at least in several prostate or lung cancer cells (Dango et al., 2011; Brickner et al., 2017). However, since the substrates for ALKBH proteins are not the major lesions induced by most clinically used alkylating agents, such effects may largely rely on the addiction to an ALKBH-dependent repair pathway in specific tissues or organs (Dango et al., 2011). Nevertheless, considering that ALKBH2 or ALKBH3 is found to be overexpressed in many types of cancer, including

prostate adenocarcinoma, non-small cell lung carcinoma, and head and neck cancer, chemotherapy developed from ALKBH-dependent alkylation repair may be promising (Konishi et al., 2005; Gilljam et al., 2009; Tasaki et al., 2011; Pilžys et al., 2019). Additionally, Stefansson et al. (2017) suggested that promoter methylation status of the *ALKBH3* gene in several breast cancers correlates with poor prognosis. In addition, a positive correlation exists between the promoter methylation and cellular N^3meC levels in several breast cancer cell lines, suggesting a potential direction for alkylation chemotherapy (Stefansson et al., 2017). However, unlike the well-established role for *MGMT* promoter methylation in glioblastoma chemotherapy, direct evidence to show the promoter methylation status of the *ALKBH* gene in guiding the cellular response to alkylating agents is still missing.

Another key predictor for ALKBH-dependent repair efficiency in cells is 2-hydroxyglutarate (2-HG) (Wang et al., 2015; Chen et al., 2017). Oncometabolite 2-HG generated in isocitrate dehydrogenase (*IDH*) mutant cells has been shown to inhibit the enzyme activity of ALKBH3 and ALKBH2 in vitro (Wang et al., 2015; Chen et al., 2017). Consistently, *IDH* mutant glioma cells exhibit defects in reversal of N^1meA lesion, resulting in hypersensitivity to the S_N2 alkylating agent MMS (Wang et al., 2015). It is notable that *IDH* mutant glioma cells are also hypersensitive to nitrosourea agents including 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), suggesting a broader protective ability of the IDH enzyme against alkylation lesions. Tran et al. (2017) suggested that cells depleted of glutamine, the precursor of 2-OG, generate more cellular N^1meA/N^3meC lesions, which will ultimately evolve into DSBs. However, other effects elicited by depletion of glutamine in cells, such as an imbalance in nucleotide metabolism, may also contribute to DNA damage (Fu et al., 2019). Nevertheless, this study indicates new possibilities for using the glutaminase inhibitor 6-diazo-5-oxo-L-norleucine (DON) clinically to sensitize cancer cells, particularly cancers with ALKBH overexpression, to alkylating agents (Tran et al., 2017).

6 A lesion more than a lesion

On the other hand, DNA methylation is an essential epigenetic control mechanism in mammals. Especially, the methylation of the fifth carbon of cytosine produces 5-methylcytosine (5mC), often referred to as the "fifth base" of the DNA code. 5mC is regulatable and programmable and can thus affect genetic information (Luo et al., 2018). It is interesting to consider whether other DNA adducts may also play a similar role as epigenetic markers in gene transcription or translation. Ample clues exist in the RNA context, where N^1meA or N^3meC, generated either enzymatically or non-enzymatically (Hori, 2014; Kietrys et al., 2017), can block translation and potentially induce ribosomal miscoding (Safra et al., 2017; Zhao et al., 2017; Zhang and Jia, 2018). Profiling of the N^1meA modifications in messenger RNA (mRNA) suggests that they are mostly enriched in the 5'-untranslated region (5'-UTR), a position opposite to that of the well-established regulatory RNA mark N^6-methyladenine (N^6meA) (Dominissini et al., 2016; Li et al., 2016; Zhao et al., 2017). Such a discrepancy in position may indicate a functional compensation between N^6meA and N^1meA in RNA. This notion has been further strengthened by the overlap recognition of N^6meA and N^1meA through YT521-B homology domain family 2 (YTHDF2) (Wang et al., 2014; Dai et al., 2018; Seo and Kleiner, 2020). Other studies suggested that ALKBH3 regulates transfer RNA (tRNA) stability through regulating N^3meC in tRNA (Ueda et al., 2017; Chen et al., 2019). However, the missing information on a bona fide methyltransferase for N^1meA or N^3meC in DNA is an obstacle to understanding the regulatory role of N^1meA or N^3meC. Nevertheless, the regulatable ALKBH-dependent repair mechanism still provides cells with the means to orchestrate the adducts' profile across the genome. Ideally, the regulatory scenario may present in certain tissues which are under persistent exposure to chemotherapeutic alkylators or where N^1meA/N^3meC is used as a potential biomarker. Future development of related genomic techniques and discovery of more participants may help reveal the potential physiological function of these DNA adducts.

7 Concluding remarks

In recent years, tremendous progress has been seen in the understanding of DNA alkylation damage repair. It is important to note that multiple layers of

complexity need to be incorporated to get a more complete understanding of the biological effects of DNA alkylation. DNA alkylation responses are variable between cells, tissues, and individuals; therefore, the responses of tumors to alkylating agents are often variable. A better understanding of the regulation of DNA alkylation may provide new opportunities to exploit genomic instability and formulate targeted therapy for cancer cells. Future advancement in deciphering the biological effects elicited by alkylation lesions could be explored through establishing novel proteomic or high through-put genomic technologies. Synthetic lethal screens will continue to be fruitful for developing therapeutic approaches, as well as providing insights into the interplay of repair signaling. These tasks are important because different answers have very different implications for the etiology of human DNA alkylation damage and provide key insights into developing individual chemotherapy.

Author contributions

Yihan PENG wrote and edited the manuscript; Huadong PEI designed the study and revised the manuscript. Both authors have read and approved the final manuscript. Therefore, both authors have full access to the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Yihan PENG and Huadong PEI declare they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by either of the authors.

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