DNA repair defects in colon cancer

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Defects in DNA-repair pathways lead to an accumulation of mutations in genomic DNA that result from non-repair or mis-repair of modifications introduced into the DNA by endogenous or exogenous agents or by the malfunction of DNA metabolic pathways. Until recently, only two repair pathways, postreplicative mismatch repair and nucleotide excision repair, have been linked to cancer in mammals, but these have been joined in recent months also by the damage-reversal and base-excision-repair processes, which have been shown to be inactivated, either through mutation or epigenetically, in human cancer.

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Abbreviations

AP	abasic (apurinic/apyrimidinic)
APC	adenomatous polyposis coli
ATM	ataxia telangiectasia mutated
BER	base excision repair
EXO1	exonuclease 1
G°	8-oxoguanine
HNPCC	hereditary non-polyposis colon cancer
IR	ionizing radiation
MBD4	methylated DNA binding protein 4
MGMT	methylguanine methyl transferase
MLH	MutL homologue
MMR	mismatch repair
MNNG	N-methyl-N′-nitro-N-nitrosoguanidine
MNU	N-methyl-N-nitrosourea
MSH	MutS homologue
MSI	microsatellite instability
MSI-H	high microsatellite instability
MSI-L	low microsatellite instability
MTH	MutT homologue
MYH	MutY homologue
OGG1	8-oxoguanine DNA-glycosylase
PMS	post-meiotic segregation

Introduction

The genomic DNA of all organisms is constantly modified by exogenous and endogenous reagents [1]. In addition, some pathways of DNA metabolism such as DNA replication also modify the genetic material by introducing errors into newly synthesized strands. In order for the DNA to fulfil its role as a template for transcription, or to serve as the genetic blueprint that is passed onto the next generation, the cells of all organisms have evolved highly sophisticated and efficient machineries that maintain the integrity of their genomes. It could logically be anticipated that the malfunction of any repair pathway, be it damage reversal, base excision repair (BER), nucleotide excision repair, mismatch repair (MMR) or recombination repair [1], would lead to an increased frequency of mutations and thus to cancer in mammals. However, this does not appear to be the case: only a few genes that encode DNA repair enzymes have been shown to be mutated in human malignancies to date.

Malfunction of MMR in humans was first identified in 1993, in tumors of the colon, endometrium, ovary and other organs targeted by the hereditary non-polyposis colon cancer syndrome (HNPCC) (see [2–6] for recent reviews). Since that time microsatellite instability (MSI), the hallmark of MMR deficiency, has also been detected in many sporadic colon tumors, where it appears to be linked to a transcriptional silencing of the *hMLH1* (where MLH stands for MutL homologue) gene [7]. The reasons underlying the tissue tropism of MMR malfunction are unclear. The principal task of MMR is to remove nucleotides that have been misincorporated into the newly synthesized strand by the replicative DNA polymerase and that have escaped detection by the proofreading activity of this enzyme complex. However, the MMR system also appears to be involved in post-replicative DNA-damage signaling, and it is this role that might help explain why the transformation process linked with MMR defects preferentially affects cells of rapidly proliferating tissues such as the colonic epithelium. This topic will be the major subject of discussion in the following paragraphs.

Recently, two more DNA repair enzymes have been implicated in colon cancer in humans. First, the gene encoding methylguanine methyl transferase (MGMT), a protein that removes methyl and other small alkyl groups from the O^6-position of guanine (reviewed in [8]), has been shown to be transcriptionally silenced in some colon tumors (reviewed in [9]). The second example is *MutY homologue* (*MYH*) [10], a homologue of the *E. coli MutY* gene, which encodes a DNA-glycosylase responsible for the removal from DNA of adenines mispaired with 8-oxoguanine (G°). *MYH* mutations have been identified in patients with multiple colorectal adenoma syndrome [11••,12••]. Most recently, disruption of the murine

methylated DNA binding protein 4 (*Mbd4*) gene, which encodes a DNA-glycosylase that removes thymine and uracil from mispairs with guanine [13,14•], was shown to result in an increased frequency of intestinal polyps in $Mbd4^{-/-}Apc^{Min/+}$ mice [15••,16].

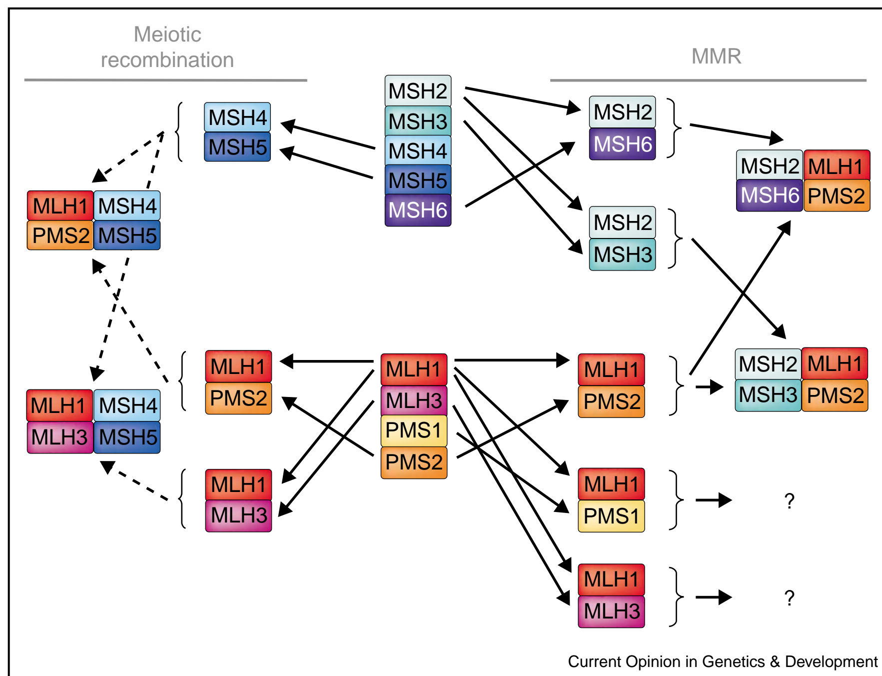
All this evidence points to a link between DNA repair, transcriptional silencing and cancer, where the MMR system plays a pivotal role. In the following paragraphs we shall attempt to elucidate the nature of this link.

Mismatch repair defects

The study of mismatch-repair defects in cancer has received a great deal of attention since the discovery mentioned above of a connection between HNPCC and germline mutations in *MMR* genes. As this topic has been extensively reviewed in the recent literature [2–4,6,17], we shall focus here primarily on work with human cell lines and transgenic mouse models.

The principal players in mammalian MMR are the homologues of the bacterial MutS and MutL proteins, which function in the form of heterodimers (Figure 1). Of these, the key initiation factors are the mismatch binding heterodimers MSH2/MSH6 (where MSH stands for MutS homologue) (MutSα) and MSH2/MSH3 (MutSβ). MutSα binds base/base mismatches and small extrahelical loops formed as a result of polymerase slippage in microsatellites, whereas MutSβ plays only a back-up role in MMR, recognizing extrahelical loops. Because MSH2 is part of both heterodimers, cell lines (Table 1) and mice (Table 2) lacking this polypeptide have a much stronger mutator phenotype than those lacking MSH6, and the mutator phenotype of MSH3-deficient lines is almost undetectable in most assays. However, the loss of both MSH6 and MSH3 brings about a phenotype that is comparable to that of MSH2-deficient cells or animals [18,19•] and the functional redundancy is therefore clearly limited to only the MSH3 and MSH6 proteins. The situation is less clear in the case of the MutL homologues, which are thought to act as 'molecular matchmakers' between the mismatch binding factor(s) and the downstream effectors of repair (i.e. the replication machinery). Although several orthologues of the MutL protein have been identified in mammals (Figure 1), only the MLH1/PMS2 (MutLα) heterodimer has been shown to function in MMR to date. No role in mammalian MMR has so far been found for the hMLH1/hPMS1 (hMutLβ) heterodimer [20] and $Pms1^{-/-}$ mice are fertile and tumor-free [21]. The MLH1/MLH3 complex [22•] was predicted to function in MMR by analogy to yeast [23], but mice with a disrupted *Mlh3* gene display a defect in meiotic recombination rather than in MMR [24•], and appear not to be cancer-prone, even though mutations in *hMLH3* in tumors with high-grade MSI (MSI-H) have been described [25]. However, $Pms1^{-/-}Mlh3^{-/-}$, $Pms2^{-/-}Mlh3^{-/-}$, $Pms1^{-/-}Pms2^{-/-}$ and $Pms1^{-/-}Pms2^{-/-}Mlh3^{-/-}$ mice should be generated, as the phenotypes of $Mlh1^{-/-}$ and $Pms2^{-/-}$ animals differ, both in cancer prone-

Figure 1



Mammalian MSH and MLH, their interactions and their involvement in MMR and meiotic recombination. Dotted arrows represent functional interactions inferred from studies in yeast or mouse systems but not demonstrated biochemically.

Table 1

Colon cancer cell lines with MMR gene defects.

Cell line	hMSH2	hMSH6	hMSH3	hMLH1	hPMS2	APC
HCT15/DLD1		*				*
LoVo	*	†	†			*
HCT116			‡	*	†	
SW48				*	†	
LS174T			Very weak expression	Very weak expression	Very weak expression	
RKO				*	†	
CO115			Very weak expression	*	†	
LS411				*	†	*
LS180				Very weak expression	Very weak expression	
GP5D	*	†	†			*
VACO481			‡		*	*

Data extracted from www.cephb.fr/gaccc, from [59–61] and from our analyses of protein expression. *Primary homozygous genetic and/or epigenetic inactivation of the gene; †Protein degradation in the absence of the heterodimeric partner; ‡Secondary inactivation by homozygous frameshifts in short repeats of the coding region.

ness (*Pms2*$^{-/-}$ animals do not develop intestinal tumors [26]) and in meiotic character (*Mlh1*$^{-/-}$ male and female mice are sterile, whereas in *Pms2*$^{-/-}$ mice only the males are sterile), and it is important to eliminate the possibility that the weak contribution of Mlh1/Mlh3 and/or Mlh1/Pms1 to MMR (if any) is not apparent in the presence of Mlh1/Pms2.

The genetic and biochemical findings summarized above help explain why most HNPCC families carry germline mutations in the *hMLH1* and *hMSH2* genes and why *hMSH6* is mutated only in atypical HNPCC families [5]. But as these mutations account for only around 70% of the HNPCC families whose tumors display MSI-H, the question arises of whether other genes linked

Table 2

Mouse models with disrupted *MMR* alleles.

Genotype	Fertility (male/female)	Tumor spectrum	MSI*	References
Msh2$^{-/-}$	yes/yes	Lymphomas, GI, skin and other tumors	yes	[62–64]
Msh6$^{-/-}$	yes/yes	Lymphomas, GI and other tumors	no	[65]
Msh3$^{-/-}$	yes/yes	GI tumors at old age	yes	[18,66]
Msh6$^{-/-}$ & *Msh3*$^{-/-}$	yes/yes	Lymphomas, GI, skin and other tumors	yes	[18,66]
Msh4$^{-/-}$	no/no	None	N/A	[67]
Msh5$^{-/-}$	no/no	None	N/A	[68]
Mlh1$^{-/-}$	no/no	Lymphomas, GI, skin and other tumors	yes	[21,26,69–72]
Pms2$^{-/-}$	no/yes	Lymphoma and sarcoma	yes	[21,26,73]
Pms1$^{-/-}$	yes/yes	None	no	[21]
Mlh1$^{-/-}$ & *Pms2*$^{-/-}$	no/no	Lymphomas, GI, skin and other tumors	yes	[26]
MMR genes & Apc		**Increased GI tumorigenesis**†		
Mlh1$^{-/-}$ & *Apc Min/+*		3×		[74]
Msh2$^{-/-}$ & *Apc Min/+*		7×		[75]
Pms2$^{-/-}$ & *Apc Min/+*		3×		[76]
Mlh1$^{-/-}$ & *Apc 1638N/+*		10×		[72]
Msh2$^{-/-}$ & *Apc 1638N/+*		14×		[77]
Msh3$^{-/-}$ & *Apc 1638N/+*		1×		[19•]
Msh6$^{-/-}$ & *Apc 1638N/+*		6×		[19•]
Msh6$^{-/-}$, *Msh3*$^{-/-}$ & *Apc 1638N/+*		10×		[19•]
MMR genes & other genes		**Relevant phenotype**		
Msh2$^{-/-}$ & *Trp53*$^{-/-}$		Embryonic female lethality; male mice viable, but succumb to tumors very early		[78]
Msh2$^{-/-}$ & *Trp53*$^{-/-}$		No embryonic lethality of female mice		[79]
Msh2$^{-/-}$ & *Rb*$^{+/-}$		As Msh2$^{-/-}$, but lymphomas developed later and were non-metastatic		[80]
Msh2$^{-/-}$ & *Tap1*$^{-/-}$		HNPCC-like tumors; no lymphomas		[63]

*MSI (high-degree only) was investigated in tumor samples, or normal tissues or culture cells; N/A: data not available; †Increase in tumor no. Apc Min/+ mice develop on average 29 intestinal tumors, whereas Apc1638N/+ mice develop on average four intestinal tumors.

to this syndrome remain to be identified. This is quite possible, but not guaranteed. The MMR process involves many polypeptides in addition to those discussed above [3]. However, most of the others — proliferating cell nuclear antigen, replication factor C, replication protein A and polymerase-δ — play key roles in DNA replication. As inactivating mutations in any one of the genes encoding these polypeptides would be lethal, if these genes were mutated in HNPCC they would have to carry missense mutations affecting amino-acid residues in domains that are only necessary for MMR and not for replication, which would be very rare. The *exonuclease 1* (*EXO1*) gene, which encodes a $5' \rightarrow 3'$ exonuclease [27•], has been reported to be mutated in HNPCC [28], but this issue needs careful analysis, as the tumors that were studied lost the mutated allele and retained the wild type one. Thus, although the role of haploinsufficiency of EXO1 in colon cancer cannot be ruled out, more evidence has to be gathered before *EXO1* is labeled as an *HNPCC* gene. It is likely, however, that we have not yet identified all the proteins that participate in MMR. Should some of these be dedicated to MMR only, rather than being involved also in other, vital DNA metabolic processes, mutations in the genes encoding these novel proteins might be found in the HNPCC families that present with MSI tumors but do not carry mutated *hMSH2*, *hMLH1* or *hMSH6* genes.

Tumors linked with germline mutations in *MMR* genes account for around 4% of all colon cancers. However, immunohistochemical screening of 1000 unselected colon tumors (Marra and Jiricny, unpublished observations) revealed that up to 12% fail to stain for MMR proteins. Baylin and colleagues were the first to show that the MMR defect in most of these tumors was linked to the silencing of the *hMLH1* promoter by cytosine methylation [7]. The tantalizing possibility that this gene might be imprinted by cytosine methylation, and that this phenomenon might segregate in HNPCC families like *MMR* mutations, cannot be ignored, following the identification of an HNPCC patient with one methylated *hMLH1* allele in DNA isolated from blood and a loss of heterozygosity of the wild-type allele in the tumor [29••]. Such cases are probably extremely rare, however, certainly when compared to the frequency of hypermethylation of the *hMLH1* promoter in sporadic colon tumors.

Although the DNA of most solid tumors is generally hypomethylated at CpG dinucleotides in bulk DNA, many CpG islands, which frequently constitute the promoters of housekeeping genes such as *hMLH1*, are hypermethylated. Many genes tend to be indiscriminately silenced by this process, but if only some are lost, and if these are involved in functions such as growth control and checkpoint control, then this will provide the tumor cells with the selective advantage that will help them to grow out (see [9] for review). The silencing of *hMLH1* will result in a mutator phenotype and it might be anticipated that it is this trait that helps cells to mutate oncogenes and tumor-suppressor genes such as *TGFβIIR*, *IGF2R*, *E2F4*, *TCF4* and other microsatellite-containing genes that have been shown to be frequently inactivated in HNPCC tumors with MSI-H (reviewed in [30]). However, although this mechanism might be at work here, an alternative scenario might also be operating. MMR-deficient cells have been shown to be resistant to certain DNA-modifying agents, such as N-methyl-N-nitrosourea, N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) and cisplatin (reviewed in [3]). This phenotype has been ascribed to their failure to arrest at the G_2/M checkpoint following DNA damage. Interestingly, recent studies show that checkpoint activation requires greater quantities of MMR proteins than are needed for mismatch correction [31,32•]. Thus, cells expressing reduced amounts of hMLH1, for example those with a partially silenced promoter, would not have a mutator phenotype but would have a defect in DNA-damage signaling and apoptotic response. In the colon, the epithelial cells lining the crypts follow a strict program of division, cell migration/differentiation and anoikis (epithelial cell exfoliation), which results in the turnover of the crypt cell population every five days or so. Cells with defective signaling and apoptosis would be expected to default on this program and might grow out into adenomas. Support for this hypothesis has come from studies with *Pms2*$^{+/-}$ mice, which were shown to develop a similar number of thymic lymphomas to wild-type animals following treatment with N-methyl-N-nitrosourea (MNU), but which displayed an increased incidence of intestinal adenomas and carcinomas. Importantly, these tumors were not MSI-H, and thus they apparently did not arise through the loss of MMR caused by MNU-induced mutations in the wild-type allele [33].

The involvement of MMR proteins in DNA-damage signaling has been the topic of much discussion recently [4,34]. The MMR system appears to be involved in activating the G_2/M cell-cycle checkpoint following treatment of cells with methylating agents [35]. MNNG treatment has been reported to activate the damage-dependent Ataxia telangiectasia mutated (ATM) kinase, and this response was at least partially dependent on an active MMR system [36]. It should be pointed out that the concentration of MNNG used in this study was very high, such that it could have caused double-strand breaks in DNA, which are known to activate ATM. It is not clear why double-strand breaks should signal via MMR. However, most recent evidence also implicates MMR in the control of the S-phase checkpoint induced by ionizing radiation (IR) [37•]. This latter phenomenon, reported to involve the ATM and CHK2 kinases, might at first sight appear rather curious, as MMR status has no — or only a very minor [38] — effect on the sensitivity of cells to IR treatment, particularly when compared to the 100-fold difference in sensitivity to methylating agents. But given

that the MMR system has been shown to address G°/adenine mispairs resulting from the incorporation of dG°MP into the newly synthesized strand during DNA replication [39•], at least one link between IR and MMR would appear to exist. In our view, the DNA-damage-signaling process mediated by MMR proteins merits careful analysis in the future.

Silencing of methylation damage reversal process

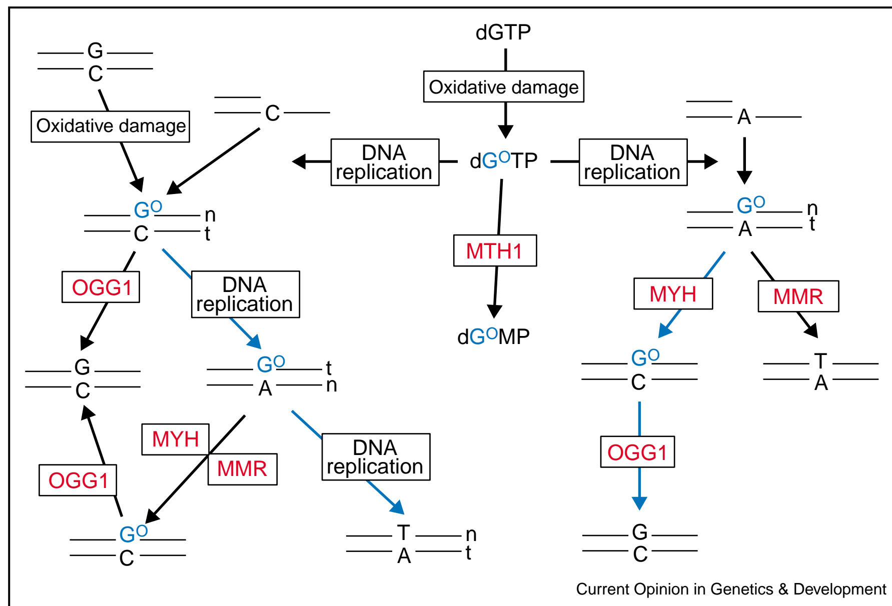
The MGMT protein plays an important role in DNA detoxification by removing small alkyl groups from the O^6-position of guanines. This modification appears to be largely responsible for the cytotoxicity of methylating agents, as cells expressing high amounts of MGMT are resistant to killing by agents such as MNU, MNNG and temozolomide, whereas cells lacking this activity are highly sensitive to these drugs [40]. Patient- and organ-specific fluctuations in the levels of MGMT are thought to be responsible for the variation in the efficacy of chemotherapy with temozolomide [41] and other simple methylating agents. Low levels of MGMT would also be expected to bring about an increase in spontaneous G→A transitions, resulting from the mispairing of unrepaired O^6-methylguanines with thymines during DNA replication. This malfunction was indeed shown to lead to an increase in activating mutations in the K-*ras* oncogene in a reporter system [42]. Recently, the *MGMT* gene was shown to be inactivated by promoter methylation in many tumor types. In colon cancers, the anticipated increase in activating K-*ras* mutations could again be observed [43]. Importantly, these tumors also carried an increased number of G → A transitions in the p53 tumor-suppressor gene [44•], which demonstrates that the effect of *MGMT* inactivation is global and that it can therefore affect any gene where this type of mutation provides the cell with a selective advantage. Interestingly, many of the tumors with silenced *MGMT* genes displayed low microsatellite instability (MSI-L) [9]. MSI is linked with the inactivation of the MMR system and primarily affects microsatellites with mononucleotide and dinucleotide repeats (MSI-H). However, in some tumors only a few of the tested markers are mutated and these tumors have therefore been assigned the MSI-L phenotype. The genetic cause underlying MSI-L is unknown; this phenotype is not linked with mutations in *MMR* genes, but the association with a silenced *MGMT* gene is interesting. One could speculate that the lack of MGMT in a cell brings about a rise in the number of unrepaired methylated bases in DNA and thus increases the number of O^6-methylguanine/thymine mispairs following DNA replication. These structures are recognized (but not repaired) by the hMSH2/hMSH6 mismatch recognition factor, and it is conceivable that if the MMR factors are sequestered as a result of this recognition then they might not be available for postreplicative MMR. In this scenario, the efficiency of DNA polymerase error repair would be reduced, which might lead to MSI-L. Support for this hypothesis could be said to lie in a study of MNU-treated *Mgmt*$^{-/-}$*Mlh1*$^{+/-}$ mice [45]: these mice produced tumors that continued to express hMLH1, and it could be argued that the reduced amount of Mlh1 expressed in the cells of these animals resulted in a faster saturation of the MMR system. However, the MSI status of the tumors was not determined in this study. This MMR-saturation hypothesis should therefore be regarded with some caution, especially as the existence of the MSI-L phenotype has recently been questioned [46]. Moreover, the link between O^6-methylguanine and MMR-mediated damage signaling should not be forgotten.

Base excision repair defects

Modification of DNA bases affects all organisms. The removal of these aberrant moieties is accomplished by several DNA glycosylases through cleavage of the glycosidic bonds. This gives rise to abasic (AP) sites. In mammalian cells, these non-informative lesions are excised from DNA by the concerted action of an AP-endonuclease, which cleaves the sugar-phosphate backbone on the 5′-side of the AP-site, and polymerase-β, which removes the baseless sugar-phosphate by β-elimination. The single nucleotide gap that is thus generated is subsequently filled in by polymerase-β. The repair process is completed by a DNA ligase, which is likely to be the DNA ligase III/XRCC1 complex [47]. Although several DNA glycosylases exist that deal with the removal of methylated bases, these modifications — with the notable exception of O^6-methylguanine — do not alter the base-pairing properties of the heterocycles and therefore are not mutagenic. Thus, malfunction of these enzymes would not be expected to lead to cancer. By contrast, hydrolytic deaminations of bases are mutagenic, as they convert cytosine, 5-methylcytosine and adenine to uracil, thymine and hypoxanthine, respectively. The same applies to modifications by reactive oxygen species, as the principal modification, G°, tends to mispair with adenine during DNA replication. It was therefore surprising that mutations in the genes encoding the principal glycosylases that deal with these types of damage, uracil DNA-glycosylase and 8-oxoguanine DNA-glycosylase (*OGG1*), have not been identified in tumors to date. Mice in which these genes have been inactivated by targeted mutations do not develop cancers either [48,49], most likely because of the redundancy of repair mechanisms that address these modifications in mammalian cells [50,51]. However, hydrolytic and oxidative damage do appear to play a role in tumorigenesis.

Deamination of 5-methycytosine in double-stranded DNA gives rise to guanine/thymine mispairs. The BER process helps revert them back to G/C, with the removal of the mispaired thymines being mediated by one of two glycosylases: thymine DNA-glycosylase [52,53] or MBD4 (MED1) [13,54]. No mutations in the former gene have been identified in human cancers to

Figure 2



Schematic representation of G° repair in mammalian cells. Oxidation of guanine residues in DNA gives rise to G°/C mispairs, which are reverted to G/C pairs by the action of OGG1 and BER. In cases where these mispairs remain uncorrected until DNA replication, polymerase-δ will insert dAMP opposite G°. Removal of A by MYH and subsequent BER will convert the G°/A mispair to G°/C, which can be addressed again by OGG1. Left uncorrected, the G°/A mispair will give rise to 50% progeny with a C → A transversion mutation. Oxidation of dGTP in the nucleotide pool will give rise to dG°TP, which is normally hydrolyzed by MTH1 to dG°MP. Incorporation of dG°MP into the newly synthesized DNA strand during replication will give rise to A/G° mispairs, which have to be corrected to A/T by the removal of dG°MP from the newly synthesized strand. This requires the involvement of the MMR system. Unsolicited involvement of MYH and OGG1 would lead to the fixation of an A → C transversion mutation. Blue arrows indicate mutagenic outcomes. n, t, newly synthesized and template DNA strands, respectively. (Adapted from [58].)

date, but *MBD4* has been shown to be mutated in colon tumors with MSI-H [55]. As this gene contains an A_{10} microsatellite tract, the observed mutations are secondary, and probably arose as a result of the pre-existing mismatch-repair defect in these tumors. However, disruption of the *Mbd4* gene in mice is associated with an increased mutability of CpG dinucleotides, the target sequences of the DNA methyl transferases that modify cytosines at these sites, and $Mbd4^{-/-}Apc^{Min/+}$ mice display an increase in C → T transition mutations in the *Apc* gene and enhanced intestinal tumorigenesis [15••,16]. However, the link between *MBD4* mutations and colon cancer is tentative and needs to be strengthened, as both MSI and $Apc^{Min/+}$ target the intestinal tract.

The latest culprit in colon cancer appears to be the *MYH* gene, which encodes a DNA glycosylase responsible for the removal of adenines that have been misincorporated opposite G° residues during DNA replication [10]. The metabolism of G° is highly complex (Figure 2) and requires the co-ordinated action of at least three oxidative-damage-specific enzymes: OGG1, which removes G° from G°/C; MutT homologue (MTH), which sanitizes the oxidized nucleotide pool by hydrolyzing dG°TP; and MYH, which removes adenine from A/G° mispairs [56]. Importantly, MYH must act only on the adenines misincorporated into the newly synthesized strand opposite G° in the template; if it also removed adenines in the template strand that mispaired with dG°MP during DNA synthesis, it would effectively fix the mutations. The action of MYH must therefore be co-ordinated with postreplicative MMR in order to prevent it from addressing the latter mispairs. Available experimental evidence supports this hypothesis: MYH appears to be associated with replication foci [57], and a direct interaction with the mismatch binding factor hMSH2/hMSH6 has also been demonstrated [58]. Recently, mutations in the *MYH* gene were identified in families with the multiple colorectal adenoma syndrome [11••,12••]. The frequency of biallelic inactivations of the *MYH* locus described in these studies appears to be so high that mutations in the *MYH* gene might turn out to be the principal cause of this syndrome.

Conclusions

MMR defects were the first DNA repair malfunction to be linked with colon cancer. More recently, inactivation of MBD4 (MED1), MYH and MGMT, either through mutations or through transcriptional silencing, has also been implicated in this malignancy. It is curious to note that all three latter enzymes have also been shown to be linked with MMR, either through a direct interaction of the peptides concerned (MBD4 and MYH) or through a

common substrate (MGMT). A detailed study of these interactions is likely to lead to a better understanding of the transformation process of the colonic epithelium.

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