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Review

How does inflammation drive mutagenesis in colorectal cancer?

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ABSTRACT

Colorectal cancer (CRC) is a major health challenge worldwide. Factors thought to be important in CRC etiology include diet, microbiome, exercise, obesity, a history of colon inflammation and family history. Interventions, including the use of non-steroidal anti-Inflammatory drugs (NSAIDs) and anti-inflammatory agents, have been shown to decrease incidence in some settings. However, our current understanding of the mechanistic details that drive CRC are insufficient to sort out the complex and interacting factors responsible for cancer-initiating events. It has been known for some time that the development of CRC involves mutations in key genes such as p53 and APC, and the sequence in which these mutations occur can determine tumor presentation. Observed recurrent mutations are dominated by C to T transitions at CpG sites, implicating the deamination of 5-methylcytosine (5mC) as a key initiating event in cancer-driving mutations. While it has been widely assumed that inflammation-mediated oxidation drives mutations in CRC, oxidative damage to DNA induces primarily G to T transversions, not C to T transitions. In this review, we discuss this unresolved conundrum, and specifically, we elucidate how the known nucleotide excision repair (NER) and base excision repair (BER) pathways, which are partially redundant and potentially competing, might provide a critical link between oxidative DNA damage and C to T mutations. Studies using recently developed next-generation DNA sequencing technologies have revealed the genetic heterogeneity in human tissues including tumors, as well as the presence of DNA damage. The capacity to follow DNA damage, repair and mutagenesis in human tissues using these emerging technologies could provide a mechanistic basis for understanding the role of oxidative damage in CRC tumor initiation. The application of these technologies could identify mechanism-based biomarkers useful in earlier diagnosis and aid in the development of cancer prevention strategies.

KEYWORDS: colorectal cancer, inflammation, DNA repair, base excision repair, mutations, deamination, oxidation.

ABBREVIATIONS

5ClC	:	5-chlorocytosine
5FU	:	5-fluorouracil
5mC	:	5-methylcytosine

8-oxoG	:	8-oxoguanine
A	:	Adenine
AID/APOBEC	:	Activation-induced cytidine deaminase/ apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
APC	:	Adenomatous polyposis coli
BER	:	Base excision repair
C	:	Cytosine
CAC	:	Colitis associated colorectal cancer
CRC	:	Colorectal cancer
Cox 2	:	Cyclooxygenase-2
DNMT1	:	DNA (cytosine-5)-methyltransferase 1
FAP	:	Familial adenomatous polyposis
G	:	Guanine
HOBr	:	Hypobromous acid
HOCl	:	Hypochlorous acid
LP-BER	:	Long patch-base excision repair
MBD4	:	Methyl-CpG-binding domain protein 4
NER	:	Nucleotide excision repair
NGS	:	Next generations DNA sequencing
NO	:	Nitric oxide
N_2O_3	:	Nitrous anhydride
NSAIDs	:	Non-steroidal anti-Inflammatory drugs
OGG1	:	8-oxoguanine glycosylase
Polβ	:	Polymerase β
SP-BER	:	Short patch-base excision repair
T	:	Thymine
TDG	:	Thymine DNA glycosylase
TMN	:	Tumor-node-metastasis
TP53	:	Tumor protein 53
U	:	Uracil
UC	:	Ulcerative colitis
UDG	:	Uracil DNA glycosylase

INTRODUCTION

Colorectal cancer is a leading cause of cancer deaths worldwide, and inflammation is thought to be an important contributing factor. However, the major mutations observed in CRC are not the type generally associated with inflammation-mediated DNA damage. This conundrum represents a current roadblock to understanding the etiology of CRC at the molecular level. The purpose of this review is to describe a new hypothesis linking inflammation and CRC mutations and to suggest how advances in next generation DNA sequencing could be used 1) to evaluate this hypothesis and 2) to develop mechanism-based biomarkers for the early detection of cancer precurors and rational strategies to reduce the incidence and mortality of CRC.

Colorectal cancer has a significant impact on human health

Colorectal cancer (CRC) is the third most commonly diagnosed form of cancer worldwide and the second leading cause of cancer-related death in developed countries with a mortality rate of about 50% [1]. The global incidence of colorectal cancer is expected to increase by 60% by 2030 [2].

In highly developed countries, both incidence and mortality from colorectal cancer have been declining. The improved survival has been linked to the removal of colon polyps, early detection efforts, and an increased awareness of modifiable risk factors such as alcohol consumption, low consumption of fruits and vegetables, high consumption of red and processed meats, obesity and physical inactivity [2]. In the US, however, the incidence of CRC is rising in persons under the age of 50 underscoring the need to further define CRC, a heterogeneous disease, at the molecular level [3].

Surgery remains the most effective treatment for CRC and the chance of survival for these patients depends largely on the stage at diagnosis. In recent clinical practice, the stage of the disease is determined according to the tumor-node-metastasis (TNM) staging, which relies primarily upon tumor histopathology, size, nodal involvement, and presences or absence of distant metastasis.

For stage I disease, five-year survival exceeds 60%; however, for stage II disease, disease-free survival is both reduced and highly variable. Patients with metastatic disease have a five-year survival of less than 10% [4]. However, current clinical practice has now also included Consensus Molecular Subtyping which better informs clinicians of selection of appropriate adjuvant therapies, predicted therapeutic response, and therefore prognosis [5].

Inflammation is a consistent theme in CRC etiology, and anti-inflammatory agents appear to have a protective effect

Rudolf Virchow first noted in 1863 that tissue inflammation was associated with the development of cancer, especially CRC [6]. Crohn and Rosenberg first reported the correlation between inflammatory bowel disease and CRC, and the elevated risk of CRC in patients with ulcerative colitis (UC) has been well documented [7-9]. Patients with colitis have an increased relative risk of developing CRC of up to 2.75-fold, with a typical latency period of decades prior to the diagnosis of CRC [10]. Inflammation is also thought to contribute to other forms of sporadic and heritable colon cancer beyond those patients with colitis [11].

Population-based studies over the past decade have shown that the decrease in CRC risk among colitis patients cannot be explained by the increased rate of prophylactic colectomies alone, and that the decreased CRC incidence among colitis patients has been attributed, in part, to chemoprevention with maintenance anti-inflammatory agents such as the 5-aminosalicylates [10, 12]. Additionally, the non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin are thought to be potentially valuable anti-tumor agents *via* inhibition of cox-2 [13, 14]. However, NSAIDs are not recommended for CRC prevention among those with average risk factors due to cardiovascular, renal, and gastrointestinal toxicities [15]. The association between diet and CRC risk is thought to be related to the "inflammatory potential" of a given diet [16]. Plant-based diets are associated with lower CRC risk whereas diets high in meat, refined grains and added sugar increase CRC risk [17]. Among post-menopausal women, adherence to American Cancer Society guidelines on diet and exercise is associated with a 52% lower CRC risk [18]. Dietary bioactive compounds including grape seed extract, curcumin, lycopene, and reseveratrol have been shown to have chemopreventive effects in part by decreasing inflammation [19, 20]. Emerging evidence suggests that diet-mediated changes in the gut microbiome and associated changes in microbiota metabolites can modulate colonic inflammation [21]. The molecular mechanisms by which dietary factors or nuticeuticals impact inflammation of the colon mucosa are as of yet unknown.

Colorectal cancer results from DNA damage events that affect key oncogenes and tumor suppressor genes

The genomic landscape of CRC is dominated by recurrent chromosomal deletions, translocations, and mutations [22]. Gene mutations in *TP53*, *APC*, *KRAS*, *BRAF*, *PIK3CA* are found in most colorectal tumors and can be used as predictive markers for patient outcome as well as selection of the appropriate chemotherapy [23]. Although a common set of genes are frequently mutated in CRC, the sequence of these changes appears to influence tumor presentation and disease progression. For example, *TP53* mutations are found in many colorectal tumors and are likely an early initiating event in colitis-associated colorectal cancer (CAC), leading to flat dysplasia, but they are a relatively late event in sporadic CRC. In contrast, *APC* mutations are considered an initiating event in sporadic CRC, leading to polyp formation, but a later event in CAC (Fig. 1) [24].

One of the first tumor suppressor genes identified in multiple human tumors was *TP53* which encodes the p53 protein. The *TP53* gene, located on chromosome 17p, is the most frequently mutated gene in human cancer (~50%). The p53 protein is comprised of 393 amino acids and functions primarily as a tetrameric transcription factor regulating the expression of stress response genes that mediate cell cycle checkpoints, DNA repair, and apoptosis. Most of the mutations observed in *TP53* are missense mutations that appear at "hotspots" which correspond to amino acid residues of the protein that interact with

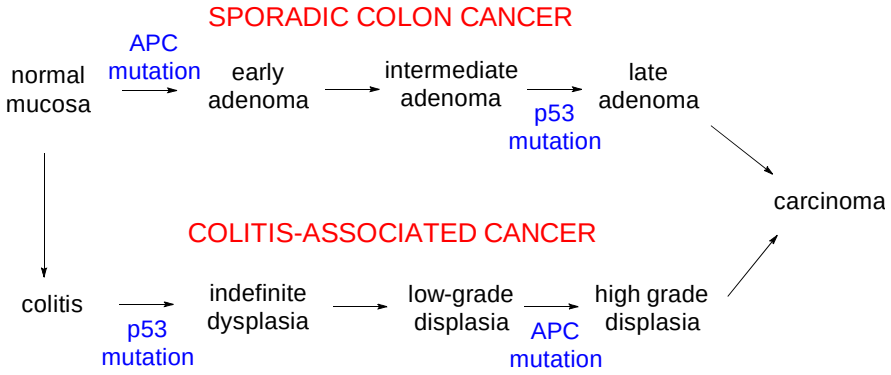

Fig. 1. Multiple pathways for the formation of colorectal tumors. This abbreviated scheme shows the suspected sequence for mutations of the APC and p53 genes in colitis-associated and sporadic colorectal cancer.

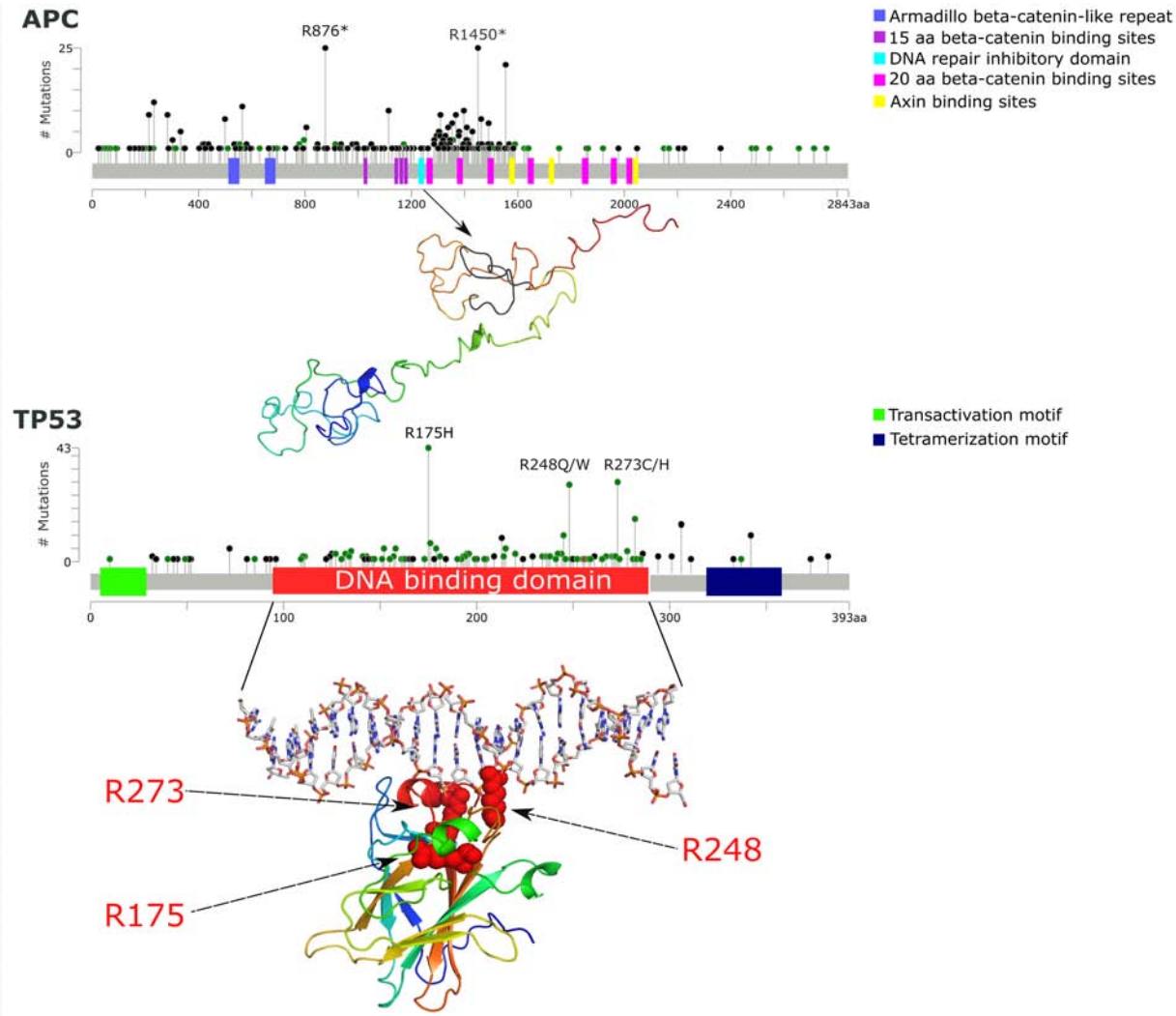

Fig. 2. Mutational hotspots observed in the APC (upper) [28-31] and p53 (lower) [39-42] genes frequently mutated in human colorectal tumors. The DNA coding sequence for each protein is shown above a three-dimensional rendering of the protein (PDB ID:1TSR).

DNA, and thus abrogate the tumor suppressor function of the p53 protein (Fig. 2) [25]. Some mutations, however, can confer a gain of aberrant function. The most predominant mutation observed in the p53 gene is a cytosine (C) to thymine (T) mutation that occurs at CpG dinucleotides located at the mutational hotspots [26, 27]. Mutation spectra for both the *p53* and *APC* genes (Fig. 2) were obtained from exome sequencing of 619 patients with colorectal cancer using the cBio Cancer Genomics Portal [28-30]. The homology model for p53 shown in Fig. 2 is based upon Cho *et al*. [31].

Another frequently mutated gene in sporadic CRC is the *APC* tumor suppressor gene. The *APC* gene is located on chromosome 5q and encodes a 2,483 amino acid protein with multiple domains (Fig. 2) involved in *wnt*-signaling, cellular adhesion, and DNA repair [32-35]. While the spectrum of mutations found in the *TP53* is dominated by missense mutations at CpG dinucleotides, the most frequent tumor-associated mutations in the *APC* gene are truncating C to T transitions at CpG dinucleotides resulting in nonsense mutations [36].

A puzzling characteristic of *APC* is its involvement in DNA repair that blocks long patch base excision repair (BER) because it is unknown why blocking a DNA repair pathway would be advantageous to a cell [37]. One line of investigation may be that an *APC* mutation renders cells resistant to 5-fluorouracil, a commonly used agent in the treatment of CRC [38]. The model for the APC protein shown in Fig. 2 was generated based upon the DNA repair domain peptide sequence identified by Jaiswal *et al*. using structure prediction and molecular dynamics refinement [37, 39-42].

Patients who have germline mutations in either *TP53* or *APC* genes are at substantially higher risk for developing CRC compared to the general population [43, 44]. Patients with Li-Fraumeni syndrome have a germline *TP53* mutation and develop several types of cancer with increased frequency, including early-onset colorectal cancer [45, 46]. Germline mutations in the *APC* gene cause familial adenomatous polyposis (FAP), an autosomal dominant cancer predisposition syndrome. If the truncated APC mutations occur in a particular region of the gene, patients can develop hundreds of adenomatous polyps and inevitably CRC [33]. These data provide evidence of a causative link in CRC tumor initiation.

The modified DNA base, 5-methylcytosine (5mC), plays an important role in the epigenetic regulation of gene transcription and also has a profound impact on mutation frequency and molecular evolution

Cytosine methylation in gene promoters is frequently associated with decreased gene transcription and aberrant promoter hypermethylation is known to result in the transcriptional silencing of tumor suppressor genes in human tumors [47]. Separate from their role in epigenetic transcriptional regulation, CpG dinucleotides are also located outside promoters and within gene coding regions, and these CpG dinucleotides have been shown to exist in the methylated form (mCpG). Jones and coworkers and Pfeifer and coworkers established that the mCpG dinucleotides in the p53 gene in human cells, independent of tissue type, are symmetrical in both strands [48, 49]. Furthermore, the location of these mCpG dinucleotides are the known “hotspots” for C to T transition mutations in many important tumor suppressor genes including p53 and APC discussed above [50]. Cytosine bases in DNA are often methylated in the CpG dinucleotide, although methylation at CpA and CpT has also been reported [51].

Harris and coworkers previously described the high proportion of C to T transitions in the *TP53* gene from colorectal tumors, and these mutations occurred predominantly at CpG dinucleotides [52]. Similar findings have been reported for mutational hotspots in multiple genes known to be mutated in CRC (Fig. 3) and other tumor-related genes including retinoblastoma [53, 54]. Indeed, the C to T transition mutation is the single-most frequent mutation found in human genetic disease, and the enzymatic methylation of the CpG dinucleotide drives tumor-associated mutations [50, 54-57]. While this observation holds true for most tumor-related mutations, C to T transitions at CpG dinucleotides in the p53 gene are found with the greatest relative abundance in human colorectal tumors [52].

The nucleophilic attack of a water molecule on a cytosine base in DNA converts it to uracil whereas an attack on a 5mC base generates thymine (Fig. 4)

The DNA of all organisms is persistently damaged by hydrolysis and oxidation [58, 59]. Among these endogenous damage events is the hydrolytic deamination of cytosine and 5mC to uracil (U) and thymine (T), respectively [60-63]. While uracil is not a normal component of DNA, the deamination of 5mC to T is a unique case where an endogenous damage reaction (hydrolysis or oxidation) to a DNA base can generate another normal DNA base. The corresponding U:G and

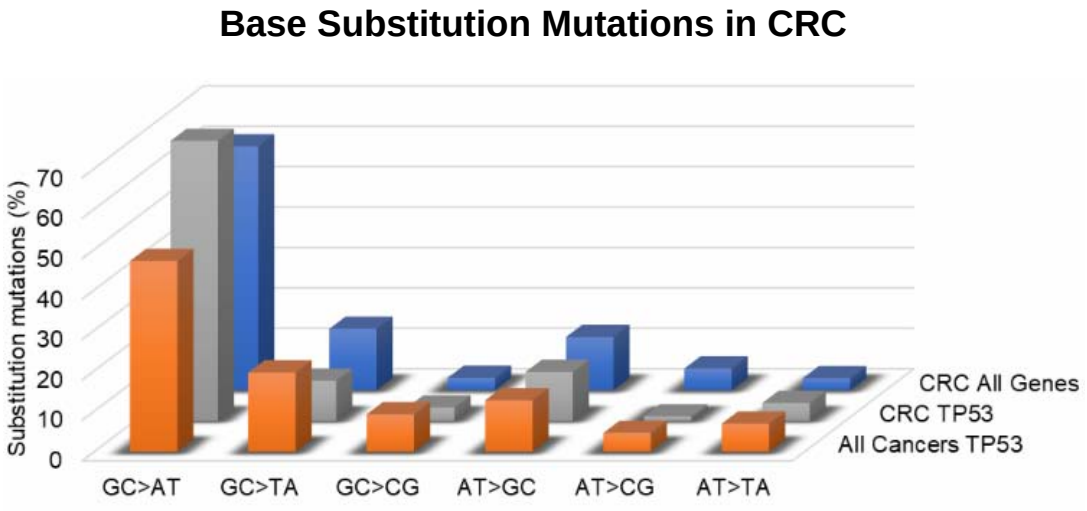

Fig. 3. Observed single-base mutations reported for CRC. Orange bars represent types of *TP53* mutations in all tumors [52], grey bars represent types of *TP53* mutations in CRC [52], and blue represents substitution mutations in all genes in CRC reported in the COSMIC database v81 [54].

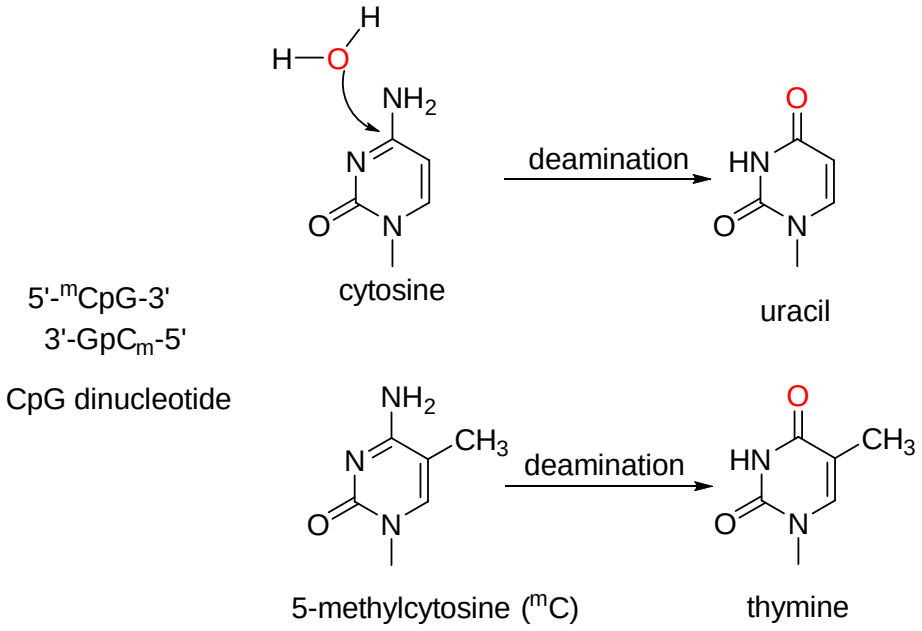

Fig. 4. The hydrolytic deamination of cytosine and 5-methylcytosine.

T:G mispairs assume a wobble-like geometry in DNA and their formation slightly destabilizes a DNA duplex [64]. When replicated by DNA polymerases, both U and T pair with A, resulting in the observed C to T transition mutations.

The substantially increased mutation frequency observed at 5mC sites in DNA cannot be attributed primarily to an increased rate of deamination. Several studies have investigated the rates of deamination of cytosine, 5mC, and various analogs. The chemical reaction is dominated by water attack on a protonated cytosine analog [65]. The electron-donating methyl group of 5mC slightly increases its pK_a, increasing the proportion of the protonated form at physiological pH [66]. However, it is known that base pair formation in duplex DNA substantially decreases cytosine deamination rates [60]. The methyl group of 5mC slightly increases DNA duplex stability and base-pair formation [67], which would tend to decrease the relative deamination rate. When considering all factors, the deamination rate of 5mC to T might be faster by a factor of two relative to the deamination rate for C to U, which is insufficient to explain the "hotspot" mutation frequency at methylated CpG sites. Therefore, k_1 in Fig. 5 would be similar for C and 5mC.

Cytosine and analogs in DNA can also be enzymatically deaminated by members of the Activation-induced cytidine deaminase/apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (AID/APOBEC) family of cytidine deaminases; however, enzymatic deamination does not explain the abundance of C to T mutations observed in CRC. The AID/APOBEC deaminases are known in B and T cell precursors where they contribute to somatic hypermutation in the immunoglobulin gene [68]. Aberrant expression of AID, induced by pro-inflammatory cytokines, has been reported in colonic epithelial cells and may contribute to C to T mutations in the *TP53* but not *APC* gene [69]. However, most members of the AID/APOBEC family of deaminases disfavor 5-substituted cytosines [70-72]. The cytidine deaminases do show sequence selectivity for runs of C's or the WRC sequence (W is A or T, R is a purine) but not specifically the CpG dinucleotide [73].

The substantial difference in mutation frequency at methylated versus non-methylated cytosine sites in DNA is due to the relative rate of repair

The deamination of cytosine in DNA generates a U:G mispair whereas the deamination of 5mC generates a T:G mispair (Fig. 4). The aberrant deaminated bases can be removed by glycosylases of the base excision repair (BER) pathway. Uracil in either U:A or U:G base pairs is efficiently removed by uracil DNA glycosylase (UDG) and all other members of the evolutionarily conserved superfamily of uracil glycosylases (Fig. 5) [74, 75]. However, UDG does not remove thymine due to a steric clash between the thymine methyl group and a Tyr residue in the pyrimidine binding pocket [76]. Thymine in a T:G mispair can be removed by the thymine DNA glycosylase (TDG) and Methyl-CpG-binding domain protein 4 (MBD4) [77, 78]. Deficiencies in either TDG or MBD4 increase the mutation frequency at CpG sites and subsequently increase tumorigenesis, strongly supporting the importance of correcting T:G mispairs for reducing CRC incidence [79-82]. Both U:G and T:G mispairs can also be corrected by mismatch repair pathways [83, 84]. It has been estimated by Schmutte *et al*. that the relative repair of U:G exceeds that of T:G by a factor of

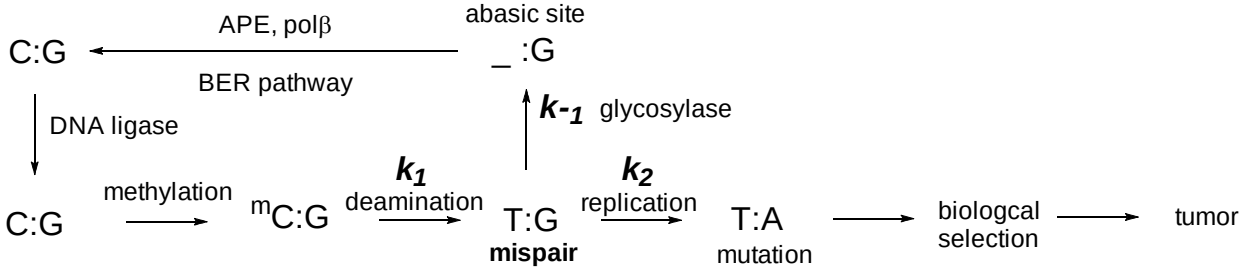

Fig. 5. Pathway for generating a C to T transition mutation in a human tumor.

nearly 6,000, representing the composite of all repair activities in human colon cells [85].

While the rates of deamination of cytosine and 5mC are roughly similar, mutational hotspots at CpG sites arise because T:G is repaired much more slowly than U:G, and therefore T:G remains in DNA longer, with a greater probability of being converted to a T:A mutation by DNA replication. As indicated in Fig. 5, the abundance of the T:G mispair in DNA is a function of it rate of formation (k_1) minus the rate of its repair (k_{-1}). Slow repair increases the chance that it will be converted to a mutation by DNA replication (k_2). If the mutation results in either an amino acid substitution at a critical site, or in a premature truncation, the mutated cell could have a selective growth advantage, promoting tumor formation (Fig. 5).

DNA mutations in colorectal cancer are often attributed to DNA oxidation damage; however, DNA oxidation predominantly generates G to T transversion mutations and not G to A (C to T) transition mutations

Oxidation damage to DNA, in particular inflammation-mediated oxidative damage, is often suggested as an underlying cause of cancer-driving mutations in colorectal cancer. The hydrolytic deamination of cytosine or 5mC results in a transition mutation (G:C to A:T) as shown in Fig. 6. In contrast, oxidation-induced mutations are generally G to T transversion mutations [86-90]. Of the canonical DNA bases, guanine is the most easily oxidized, with the formation of 8-oxoguanine (8-oxoG). An important characteristic of 8-oxoG is its ability to flip from the *anti* to *syn* conformation (Fig. 6) allowing mispairing with A during polymerase-mediated DNA replication, generating a G to T transversion mutation. While a defect in 8-oxoG repair could potentially increase mutagenesis, the repair capacity for 8-oxoG is surprisingly higher in CRC patients relative to controls [88]. Although G to T transversions are seen in colorectal cancer (Fig. 3) and other cancers, in particular in the p53 gene in hepatocellular carcinomas due to aflatoxin B1-exposure [86], DNA oxidation cannot explain the apparent increase in C to T transition mutations at CpG dinucleotides that are so predominant in colorectal cancer (Fig. 3).

Reactive nitrogen species are unlikely to explain C to T transitions at CpG dinucleotides

Chemical derivatives of nitric oxide have long been suspected to be involved in cancer-relevant mutations [91, 92]. Nitric oxide (NO), generated under inflammatory conditions, is associated with an increased frequency of C to T transitions at CpG sites at codon 248 of the p53 gene in colon tissues from patients with ulcerative colitis [93]. Nitric oxide can oxidize forming nitrous anhydride (N_2O_3) or combine with superoxide forming peroxynitrite, and both derivatives can induce deamination. Tannenbaum and coworkers have shown that N_2O_3 can cause the deamination of cytosine to uracil. However, the deamination of guanine to xanthine proceeds more rapidly [94]. When in DNA, xanthine can miscode with T, generating a C to T transition mutation, but this mutation pathway would not target CpG dinucleotides. Xanthine in DNA is also rapidly removed by several glycosylases [95]. In *E. coli*, the predominant mutation from nitrosative damage is an A:T to G:C mutation, resulting from deamination of adenine to hypoxanthine with subsequent polymerase pairing of hypoxanthine with C [96]. Schmutte *et al*. and Tamir *et al*. have argued that, because a defect in uracil glycosylase has only a minor impact on nitric oxide toxicity or mutagenesis, the appearance of G:C to A:T transition mutations following nitric oxide treatment is unlikely to induce deamination of C or 5mC [97, 98]. In the APCMin/+Msh2-/- mouse model, nitric oxide does not appear to promote colon cancer [99]. Dong and Dedon have reported only small increases in the levels of nucleobase deamination products in human TK6 lymphoblast cells exposed to toxic levels of nitric oxide [100]. While NO treatment can result in increased mutations and oxidative DNA damage the observed mutations are unlikely the result of nitrosative deamination of 5mC.

Miscoding by 5mC damage products induced by reactive halogen species are unlikely to explain the predominance of G:C to T:A mutations in human colorectal cancer

Reactive molecules are generated at sites of inflammation due to the activation of neutrophils and eosinophils. Myeloperoxidase from activated neutrophils and eosinophil peroxidase from activated

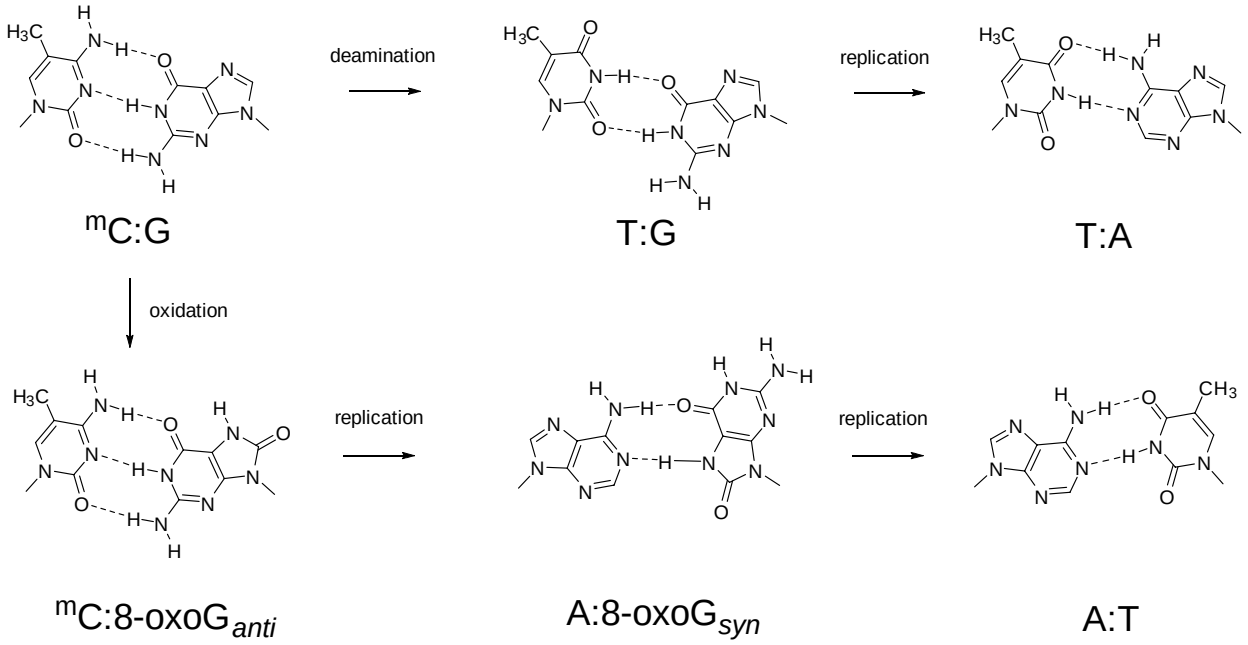

Fig. 6. Transition mutations generated from the deamination of 5mC versus transversion mutations generated by oxidation and miscoding of guanine.

eosinophils can generate HOCl and HOBr, respectively. These reactive halogen species can damage proteins, nucleic acids, and lipids and are, therefore, powerful antimicrobial agents. However, damage induced by these agents is indiscriminate and substantial collateral damage can occur to host biomolecules [101]. HOCl can react with DNA cytosine at a CpG dinucleotide generating several products including 5-chlorocytosine (5ClC) [102]. Due to the similar size of the methyl and chlorine substituents, 5ClC can act as a fraudulent epigenetic signal, facilitating the binding of proteins containing a methyl-binding domain, as well as inducing DNMT1 to methylate the newly replicated strand following DNA replication [103-105]. Mangerich *et al*. have shown that 5ClC can be found in the DNA of inflamed colon tissue; however, it is unlikely to cause C to T transition mutations at CpG dinucleotides for the reasons explained below [106]. Even though Essigmann and coworkers recently demonstrated the slight miscoding properties of 5ClC in the presence of repair polymerase polβ, it is unlikely to explain the observed mutations in CRC because the cytosine at a CpG dinucleotide is present as 5mC [107]. Cytosine can be converted to 5ClC, but 5mC cannot be converted to 5ClC [102]. Thus, because the CpG dinucleotides at the mutational hotspots are predominantly 5mC, HOCl is unlikely to be the cause of the inflammation-induced increase in C to T mutations at CpG sites, although it could cause increased transition mutations at non-methylated sites and alter epigenetics.

How might inflammation drive the predominant mutations that underlie the development of colorectal cancer?

As shown in Fig. 3, there is an unusual abundance of C to T transition mutations at CpG dinucleotides in critical genes, leading to CRC [50, 52, 56]. Although methylation does not substantially increase the rate of deamination of 5mC (Fig. 5 k_1), the deamination product, thymine, is repaired thousands of times more slowly that uracil (Fig. 5, k_{-1}). Defects in genes encoding proteins that repair the T:G mispair are associated with increased mutagenesis and tumorigenesis as discussed above. Collectively, this evidence strongly implicates the sequence ^{m}C:G to T:G to T:A shown in Fig. 5.

How would oxidative DNA damage promote the pathway resulting in a C to T transition mutation (Fig. 5)? Oxidative DNA damage causes

predominantly G to T transversions, not C to T transition mutations (Fig. 6). A mechanistic connection between oxidative DNA damage and C to T mutations would allow us to understand inflammation-induced initiation of CRC at the molecular level. A potential explanation for how inflammation could drive C to T mutations might be found in the mechanisms by which the T:G mispair is converted to the T:A mutation (Fig. 5, $\boldsymbol{k_2}$). Although $\boldsymbol{k_2}$ could occur during DNA replication as part of cell division, oxidative DNA damage would tend to decrease the rate of cell division [108]. Alternatively, the T:G mispair could be converted to the T:A mutation inadvertently during DNA repair as described below. Important in understanding this hypothesis is the recognition that human cells have multiple pathways for repairing both endogenous and exogenous DNA damage, and that these pathways might intersect or compete with unanticipated biological consequences. For instance, hydrolytic deamination would convert a ^{m}C:G base pair to a T:G mispair (Fig. 5) but if this site were damaged by oxidation before replication or repair, a T: 8-oxoG mispair would be present, leading to a competition between the repair of the T and the 8-oxoG (Fig. 7). If the 8-oxoG was first recognized, triggering a round of the BER pathway, the T in the opposing strand would be the template for the DNA repair polymerase, polβ, resulting in the conversion of the site to a T:A base pair. The slow repair and lengthy persistence of the T:G mispair and increased oxidative DNA damage would then both contribute to the C to T mutation. Alternatively, glycosylase removal of T from a T:8-oxoG mispair could result in the placement of C or A opposite the 8-oxoG.

As stated previously, guanine is the most oxidizable of the DNA bases, forming 8-oxoguanine. The rate of guanine oxidation is influenced by local DNA sequence, and the presence of an adjacent 5mC increases the rate of guanine oxidation by a factor of ~2 [109]. Once formed, the 8-oxoG mispaired with T can be removed by 8-oxoG-DNA glycosylases from humans, rodents, and yeast [110, 111] which could be converted to a T:A base pair by the BER pathway. It is yet unknown how conversion of G to 8-oxoG would alter the rate of T cleavage by TDG or MBD4. Upon the basis of previously published literature, we can propose an alternative pathway by which oxidation can induce C to T mutations, involving oxidation of the G of the T:G mispair followed by BER removal and repair of the 8-oxoG.

Both the deamination of 5mC and the oxidation of G occur every day in every living human cell. However, they are rare events among the 10^9 base pairs in the human genome, so the likelihood that both would occur simultaneously at the same bases pair is low. Alternatively, oxidative damage to the DNA strand opposite the deaminated T, but several bases away, could similarly facilitate conversion of the T:G mispair to a T:A mutation as shown in Fig. 8. During BER, either a single nucleotide (short-patch or SP-BER) or multiple nucleotides (long patch or LP-BER) can be removed and replaced (Fig. 8). The option to execute SP or LP-BER is dependent upon the nature of the DNA damage and the presence of BER-interacting proteins.

In this example, any lesion in the lower strand triggering BER located on the right side (3') to the mispaired T could initiate LP-BER. The lesion could be damage to T, C, A or G and could result from reactive oxygen, nitrogen or halogen species. Furthermore, potential damages also include damage to the deoxyribose as both oxidation and reduction of the sugar moiety at the damaged site force repair *via* the LP-BER pathway [112-115]. Several additional protein activities are required for LP-BER, and these activities cleave the DNA flap generated by strand-displacement synthesis, or otherwise remove damage at the site that would prevent ligation of the repair gap and completion of the BER cycle [114, 116]. More recently, an additional LP-BER sub pathway has been identified that cleaves 9 bases to the 5' side of a BER substrate lesion [117]. Therefore, DNA damage in the complementary strand, within several bases of the mispaired T in either direction, could result in conversion of the T:G mispair to a T:A mutation by BER.

Repair of multiple lesion types by BER, including those induced by oxidative DNA damage, could therefore promote C to T mutations. However, additional pathways, including nucleotide excision repair (NER) could also be involved. Recently,

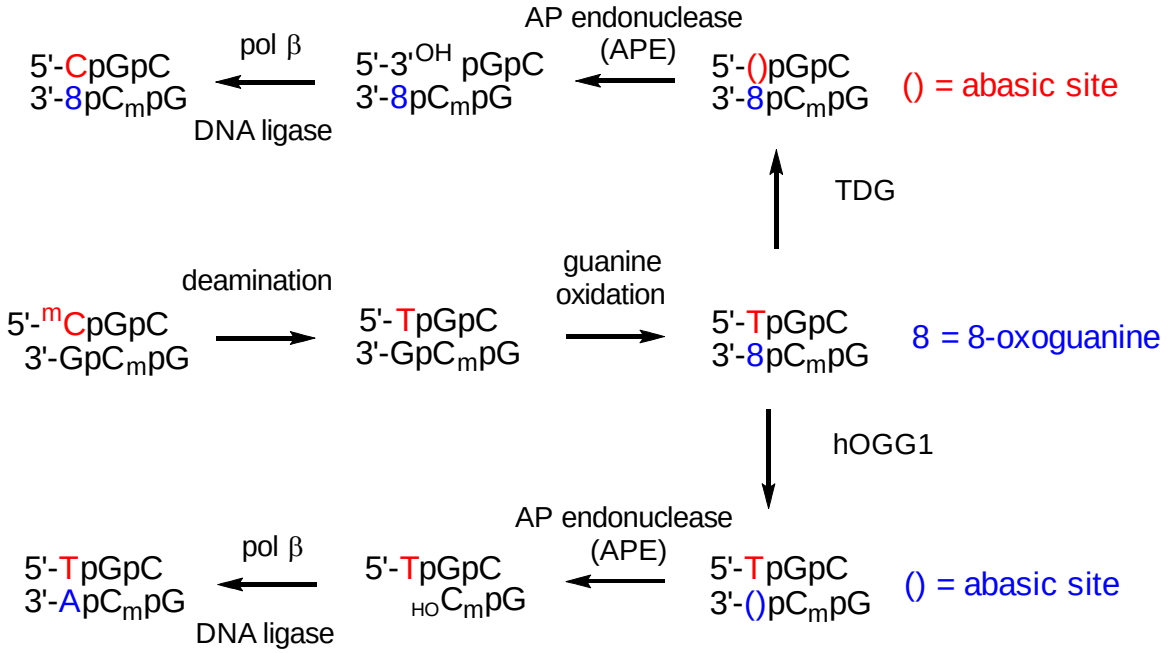

Fig. 7. Competition between short-patch BER repair pathways. In the above example, the T of a T:8oxoG mispair can be removed by TDG or MBD4 and replaced by C (upper pathway) or the 8oxoG can be removed by hOGG1 and replaced by A (lower pathway).

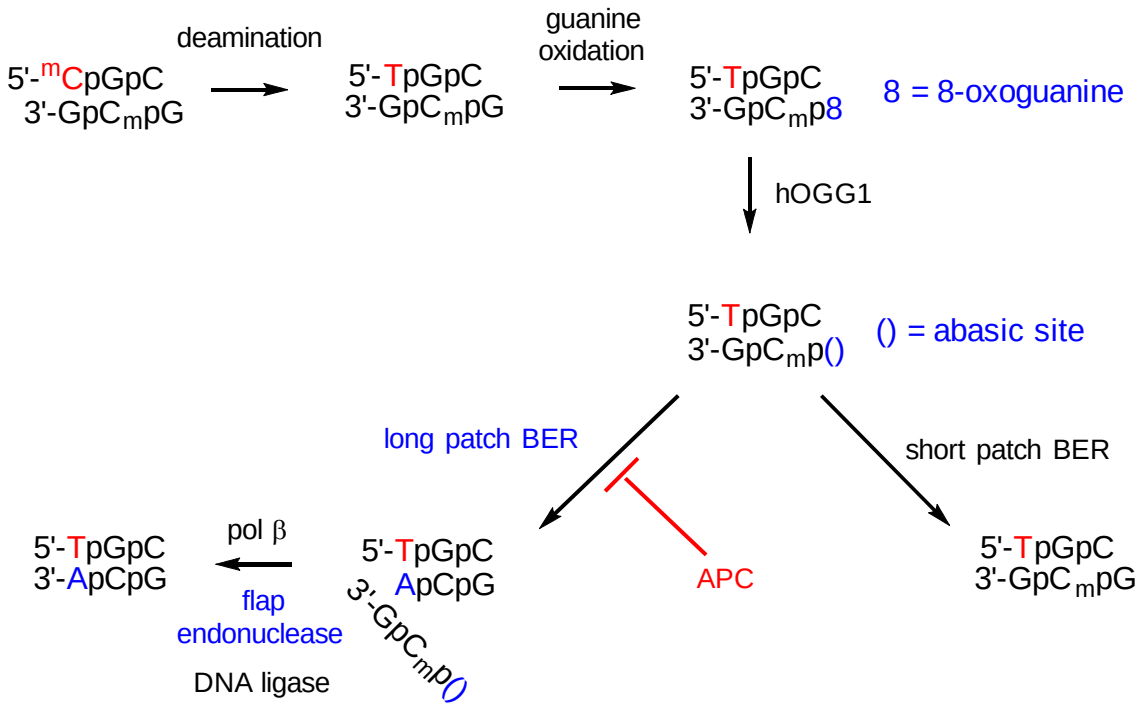

Fig. 8. The conversion of a T:G mispair to a T:A mutation by guanine oxidation followed by long-patch base excision repair. Long-patch BER could convert the T:G mispair to a T:A mutation; however, this pathway is blocked by the APC protein.

Shafirovich *et al*. demonstrated that the further oxidation derivatives of 8-oxoG, spiroiminihydantoin (Sp) and 5-guanidinohydantoin (Gh), are substrates for NER [118]. Therefore, the removal of a 25-30 nucleotide repair patch opposite a mispaired T, followed by repair synthesis would convert a T:G mispair to a T:A mutation. The recent work of Sahfirovich *et al*. reinforces the concept that various DNA repair pathways can compete for repair of the same lesion, with differing outcome depending upon the DNA repair background of a particular cell.

Multiple DNA lesions can occur in DNA and the "clustering" of this damage has been the subject of research in the area of ionizing radiation damage and repair [119-121]. Predicting the outcome of DNA repair when multiple pathways compete for the repair of a single lesion is difficult, but predicting the outcome when multiple pathways compete for more than one DNA lesion is even more complex. Presumably, an advantage of LP-BER is that multiple damaged DNA bases in proximity on a single strand could be removed and replaced by a single round of LP-BER. LP-BER is known to protect cells from alkylation damage [122]. Surprisingly, patients with CRC who have *APC* mutations do not benefit from 5-fluorouracil (5FU)-based chemotherapy [38]. As discussed previously, an additional function of the APC protein is to inhibit LP-BER [37, 123]. What would be the physiological advantage of inhibiting LP-BER? In 5FU-treated cells, both 5FU and uracil accumulate in the DNA and both are removed by BER. The inhibition of LP-BER by the APC protein places APC-normal cells at a disadvantage in the face of 5FU therapy. One possible advantage of inhibiting LP-BER is to prevent a lethal double strand break when two BER lesions occur in opposing DNA strands within 7 base pairs. Another potential advantage of APC-inhibition of LP-BER is to prevent the conversion of a T:G mispair to a T:A mutation as illustrated in Fig. 8. Consideration of the complexity resulting from simultaneous multiple DNA damage events could reveal critical aspects of APC function and lead to potential pharmacological approaches to exploit *APC* mutations.

When do cancer-driving mutations occur, and how long before a tumor occurs can they be detected?

Current genetic analysis of tumors seeks to characterize critical mutations because the mutations are of diagnostic and prognostic value and can be used to stratify patients with respect to treatment strategies. Several studies have reported the presence of p53 mutation in colorectal cancer [124-126]. However, it is still unknown when during tumor development these mutations occur and at what point can the mutations be detected. Colorectal tumorigenesis begins when a single cell acquires a mutation inactivating a critical tumor suppressor that provides a growth or survival advantage. In the case of sporadic CRC, it has been estimated that the time interval between an initial, critical mutation in the APC gene and the development of CRC could be 25 years [127].

In CAC, a critical initiating mutation frequently occurs in the *TP53* gene. Initially, one copy of the gene becomes mutated while at some later time, the other copy is deleted. For some time, multiple laboratories have shown the presence of *TP53* mutations in noncancerous tissues. In 1993 and 1994, Yin *et al*. and Brentnall *et al*. reported that mutations in exons 5-8 of the *TP53* gene could be detected in noninvasive dysplasias adjacent to tumors, suggesting the existence of a "field effect" that could be of potential prognostic importance in the context of ulcerative colitis (UC) [128, 129]. A retrospective study in patients with long-standing UC showed the presence of *TP53* mutations in pre-colectomy biopsies that preceded colectomy by 14 years, indicating the long period of time over which critical mutations can be detected in human tissues before tumors can be identified [130]. Lang *et al*. demonstrated the DNA isolated and sequenced from lavage solution obtained at surveillance colonoscopy of patients with long-standing UC contained mutations in the *TP53* gene [131]. Several additional studies have reported on the presence of *TP53* missense mutations in non-neoplastic mucosa [35, 132, 133].

Several years ago, Hussain *et al*. showed by DNA sequencing that colonic tissues of ulcerative colitis patients, but not normal controls, contained

low levels of C to T mutations at codon 248 of the *TP53* gene [134]. In this study, mutated DNA was enriched by endonuclease cleavage prior to amplification. Their analysis revealed C to T mutations at the first position of codon 248 (Arg to Trp), a methylated CpG site, in inflamed tissue, but not in non-lesional controls. Surprisingly, similar levels of C to T mutations were also observed at the last position of codon 247, an unmethyated, non-CpG site resulting in a silent mutation. Nitric oxide synthase levels were also higher in tissues from UC patients, relative to controls, suggesting oxidative stress in the etiology of the *TP53* mutations. Jenkins *et al*. showed similar mutational findings in primary human foreskin fibroblasts and the HGC-27 human gastric cancer cell line treated with hydrogen peroxide [135]. Heinzlemann *et al*. showed both silent and missense mutations in DNA extracted from whole-gut lavage in patients with chronic inflammatory bowel disease [136]. Yoshida *et al*. demonstrated heterogeneous missense and silent mutations within microdissected single crypts from colectomy specimens obtained from patients with long-standing UC [137]. The significance of these findings is that sensitive methods can be developed to measure critical mutations in inflamed human tissues. Although synonymous mutations are frequently unreported in sequencing studies, silent mutations do reflect damage and repair events in cells, and when compared to transition mutations at critical sites, could be used to indicate if biological selection had begun in a particular tissue.

Future perspectives

Next generation DNA sequencing (NGS) provides tools essential for measuring low-frequency mutations within complex, heterogeneous samples

Next generation DNA sequencing (NGS) technology is revolutionizing the flow of genetic information into clinical practice [126]. NGS is a high-throughput technique that can provide large-scale sequencing of entire genomes, and it can also provide “depth” in sequencing analysis. In NGS, single DNA molecules within a mixture are sequenced which allows examination of sequence heterogeneity within biological samples. Traditional Sanger DNA sequencing cannot reveal the presence of mutated sequences that occur in a mixed DNA population at a frequency of less than 20%. In contrast, next-generation deep-sequencing can measure extremely rare mutations [138]. Both mosaicism in normal tissues as well as intra-tumor heterogeneity have been measured with NGS techniques [139-144]. The measurement of rare mutations can assist in patient treatment stratification and testing for minimal residual disease. Current limitations in the sensitivity of these methods include PCR amplification errors, sequencing calling errors and apparent errors resulting from damage to the DNA. Several laboratories are working toward modifications to further increase the sensitivity of the measurement of minor or mutated alleles [145-148]. In addition to experimental modifications, computational approaches are also being applied to reduce current limitations with NGS deep-sequencing approaches [149-152].

Next-generation DNA sequencing methods are sensitive to DNA damage

Emerging NGS methods are increasing sensitivity; however, challenges have arisen due to apparent artifacts generated by DNA damage. The use of NGS methods to sequence ancient DNA from a Neanderthal and a mammoth revealed patterns of DNA damage that could be attributed to both deamination of adenine and cytosine in DNA and depurination, an endogenous reaction know to occur persistently in DNA [153, 154]. Studies on more contemporary DNA samples have similarly revealed the contribution of both cytosine deamination and 8-oxoguanine formation to apparent “artifactual” mutations observed with NGS methods [155-163]. The presence of uracil in DNA resulting from cytosine deamination would generate C to T mutations, whereas the oxidation of G to 8-oxoG would generate G to T mutations in NGS studies. Chen *et al*. have examined strand bias in mutations arising from damaged bases and have devised an approach to measure the magnitude of damage in a given DNA sample upon the basis of the observed strand bias. When using this approach to examine mutations in the ATCC database, Chen *et al*. concluded that a substantial number of the DNA samples did contain damaged DNA [163].

Thousands of endogenous DNA damage events occur daily in all living cells, and factors thought to increase DNA damage artifacts include thermal cycling, extraction of DNA from paraffin-embedded formalin-fixed tissues, and sonication.

An approach to minimize the impact of DNA damage in deep-sequencing studies involves incubation of the DNA repair enzymes, including uracil DNA glycosylase (UDG), to remove uracil arising from cytosine deamination, and 8-oxoguanine glycosylase (OGG1) to remove 8-oxoguanine. The abasic sites generated by the glycosylases generally block DNA replication, and therefore "damaged" DNA is eliminated from the subsequent amplification process. A remaining challenge with this approach is that the UDG removes uracil but not thymine arising from 5mC deamination. Therefore, C to T mutations at non-methylated CpG sites can be corrected, but those at methylated CpG hotspot sites cannot be eliminated by current methods [155, 162].

Improvements in NGS sequencing could allow measurement of DNA damage and repair at sequence resolution

Interestingly, the "damage" to DNA giving rise to "artifacts" in NGS sequencing also generates mutations that underlie the development of human cancer. Would it be possible to use NGS methods to measure the formation and repair of damage to the human genome at sequence resolution? Efforts from the Hesselberth and Sancar laboratories have presented evidence that the treatment of damaged DNA with repair enzymes, followed by deep-sequencing can reveal locations of DNA damage at sequence resolution [164, 165]. We propose that such methods might be generally applied to studies of DNA damage and repair and potentially lead to clinically useful assays.

For example, the sequence of events leading from a 5mC:G base pair to a T:A mutation are shown in Fig. 5. We are proposing here that oxidative damage does not increase the deamination of 5mC to T (k_1 in Fig. 5), but rather increases the conversion of the T:G intermediate base pair to the T:A mutation (k_2 in Fig. 5) due to oxidative-damage induced repair of the opposing strand. Currently, the T:G intermediate cannot be measured by available methods. The presence of the T:G mispair at codon 248 of the *TP53* gene would inhibit cleavage by the MspI restriction endonuclease, and therefore the method of Hussein *et al*. could not distinguish between the T:G mispair and the T:A mutation.

The formation of the T:G mispair or the T:A base pair at a CpG site (Fig. 5) would result in the measurement of a C to T mutation at that location by NGS methods. If a suitable glycosylase could be found to cleave the mispaired T from a T:G mispair, the "frequency" of C to T mutations at that site would be diminished. The magnitude of the decrease would indicate the relative abundance of the T:G mispair versus the T:A bases pair. With this method, the location and frequency of T:G intermediates could be measured, allowing resolution of the rate of formation (k_1), repair (k_{-1}) or replication to the T:A mispair. Glycosylases that could remove T from a T:G mispair are known; however, these glycosylases are much less robust than UNG. Undoubtedly, a suitable enzyme will be identified or engineered in the near future for such a purpose. The oxidation of G to 8-oxoG results in G to T sequence changes, and therefore, NGS sequencing before and after incubation of a DNA sample with an 8-oxoG glycosylase could be used with existing NGS methods to measure the formation and repair of 8-oxoG in and around CpG mutational hotspots.

Extension of current clinical assays to examine DNA damage and repair in clinically relevant settings

The role of DNA sequence changes in oncology is of growing importance for both early detection and prognostic stratification [166]. While the measurement of mutations in patient tissue biopsy materials will continue to have an important role, mutation measurement in cell-free DNA is increasingly more frequent [167]. With respect to CRC, fecal testing has also emerged as a non-invasive screening approach [168, 169]. One such test, Cologuard, has been approved by the FDA, and this test measures mutations in *KRAS* and *NDRG4* as well as methylation changes in the *BMP3* promoter [170]. While this test has been shown to detect CRC in asymptomatic patients at normal risk, it is not yet successful in measuring the majority of precancerous lesions and produces substantial false positives.

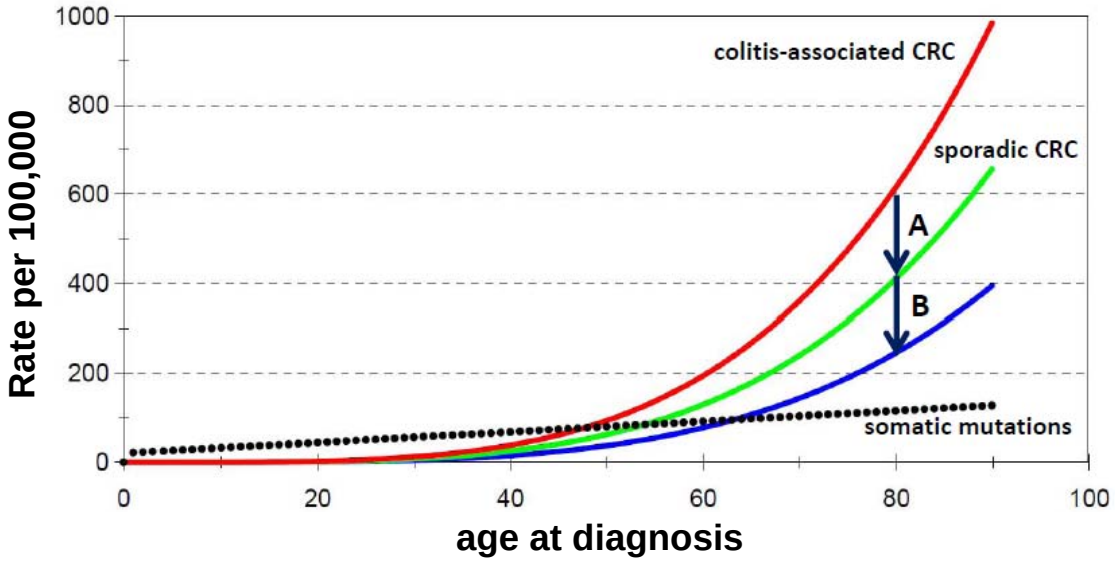

Fig. 9. Colorectal cancer incidence increases with age. Colorectal cancer occurs more frequently in patients with a history of colitis (red line) than in patients with sporadic CRC (green line) [173]. Recent developments in treating the underlying inflammation in patients with colitis have reduced CRC incidence to that of patients without colitis (A). Further developments in monitoring inflammation and DNA damage history could reduce CRC incidence (blue line, B). Somatic mutations in tissues of patients diagnosed with colorectal cancer increase with age (dotted line, trend line from Tomasetti *et al.*) [171]. Measurement of mutations (synonymous and passenger) might provide a history of DNA damage in a given tissue or alternative source of cell-free DNA.

Tomasetti *et al*. have reported that half or more somatic mutations in cancers of self-renewing tissues originate prior to tumor initiation [171]. Therefore, the measurement of these apparent "passenger mutations" in a DNA target region, such as exon 7 of the *TP53* gene could provide an index of the damage history, related to both patient age and history of inflammation. The increase in known "driver mutations" relative to silent mutations would indicate that biological selection had been initiated. The application of NGS methods to circulating and fecal DNA testing could reveal DNA damage history, placing the potential significance of selected mutations in perspective.

In Fig. 9, we show the hypothetical age-specific incidence of CRC as a function of age. The incidence of both sporadic (green line) and colitis-associated CRC (red line) increase with age, but the rate of increase is steeper for CAC [172, 173]. Recent advances in controlling inflammation in CAC patients have resulted in a dramatic decline in the incidence of CRC in this group, reducing incidence to near that of patients without colitis (A). If the underlying mechanisms driving DNA damage and mutation in CRC were revealed and suitable biomarkers identified, it is plausible that CRC incidence overall in the population could be significantly decreased (blue line).

Tomassetti *et al*. measured exome mutations in colorectal tumors and established that mutations increase with age at diagnosis for colorectal cancer [171]. Although there was substantial scatter in the data, a trend line was established and is indicated by the dotted line in Fig. 9. We propose that deep-sequencing of the region around codon 248 of the *TP53* gene, examining all mutations (synonymous, nonsynonymous, passenger, driver) could reveal differences between patients with and without colitis or CRC and could be used to estimate the damage history of a tissue. Sequencing would reveal the relative frequencies of C to T mutations at methylated and unmethylated sites, as well as indicate the frequency of G to T mutations, a marker of oxidative DNA damage. These methods could be applied to tissue DNA or cell-free DNA. Population studies could establish the age-adjusted slope of the curve,

allowing the creation of a DNA damage index, from which the CRC risk for a specific patient could be estimated. As with recent progress with colitis patients, interventions including nutrients or pharmaceuticals might favorably modify a patient's DNA damage index. Such strategies would then form the basis of rationale approaches to reduce CRC incidence and mortality.

SUMMARY

Mutations in key genes underlie the conversion of normal cells into tumors. In colorectal cancer, a subset of tumor suppressor genes and proto-oncogenes are persistently mutated. Developments in NGS methods are allowing the measurement of minor mutated alleles in heterogeneous DNA samples, including tumor and non-tumor tissues, with increasing sensitivity. These developments have immediate application in identifying levels of pre-cancerous lesions in otherwise normal tissue and in identifying rare alleles of important prognostic implications in identified tumors. Such methods are currently being extended to examine DNA damage and repair in human DNA samples at sequence resolution, and they could be used to test a model proposed here to explain how inflammation might increase C to T mutations at CpG dinucleotides, a recurrent finding in CRC and other tumor types. Beyond testing mechanisms, emerging NGS methods could be used to examine the effects of genetic defects in DNA repair on the rates of DNA damage, repair, and mutation. Ultimately, such methods could measure the DNA damage history of a given tissue. By comparing levels of silent mutations to tumor-driving missense mutations, it might be possible to estimate how far a given tissue had evolved toward the development of a tumor.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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