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EDITORIAL

Epigenetic reduction of DNA repair in progression to gastrointestinal cancer

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Abstract

Deficiencies in DNA repair due to inherited germ-line mutations in DNA repair genes cause increased risk of gastrointestinal (GI) cancer. In sporadic GI cancers, mutations in DNA repair genes are relatively rare. However, epigenetic alterations that reduce expression of DNA repair genes are frequent in sporadic GI cancers. These epigenetic reductions are also found in field defects that give rise to cancers. Reduced DNA repair likely allows excessive DNA damages to accumulate in somatic cells. Then either inaccurate translesion synthesis past the un-repaired DNA damages or error-prone DNA repair can cause mutations. Erroneous DNA repair can also cause epigenetic alterations (*i.e.*, epimutations, transmitted through multiple replication cycles). Some of these mutations and epimutations may cause progression to cancer. Thus, deficient or absent DNA repair is likely an important underlying cause of cancer. Whole genome sequencing of GI cancers show that between thousands to hundreds of thousands of mutations occur in these cancers. Epimutations that reduce DNA repair gene expression and occur early in progression to GI cancers are a likely source of this high genomic instability. Cancer cells deficient in DNA repair are more vulnerable than normal cells to inactivation by DNA damaging agents. Thus, some of the most clinically effective chemotherapeutic agents in cancer treatment are DNA damaging agents, and their effectiveness often depends on deficient DNA repair in cancer cells. Recently, at least 18 DNA repair proteins, each active in one of six DNA repair pathways, were found to be subject to epigenetic reduction of expression in GI cancers. Different DNA repair pathways repair different types of DNA damage. Evaluation of which DNA repair pathway(s) are deficient in particular types of GI cancer and/or particular patients may prove useful in guiding choice of therapeutic agents in cancer therapy.

Key words: Epigenetic; DNA damage; DNA repair; DNA repair deficiency disorders; Epimutation; Genomic instability; Germ-line mutation; MicroRNAs; Precancerous conditions; Gastrointestinal cancer

Core tip: The primary cause of cancer is DNA damage. DNA damage leads to replication errors and erroneous repair, and can result in driver mutations and epimutations. While germ-line mutations in DNA repair genes cause cancer-prone syndromes, mutations in DNA repair genes are infrequent in sporadic gastrointestinal cancers. However, reduction of DNA repair proteins due to epigenetic repression of DNA repair genes is

very frequent and can cause early steps in sporadic cancers. Evaluation of which DNA repair pathway(s) are deficient in particular types of GI cancer and/or particular patients may prove useful in guiding choice of therapeutic agents.

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REDUCED DNA REPAIR INCREASES CANCER RISK

Germ-line mutations in DNA repair genes cause increased risk of GI cancers. Examples are given in Table 1.

About 5% to 10% of all types of cancers are due to hereditary cancer syndromes[12]. Two reviews on hereditary cancer syndromes list 48 and 55 such syndromes[12,13]. Mutation in any of 37 DNA repair genes, including those listed in Table 1, can cause an hereditary cancer syndrome[14]. That hereditary cancer syndromes are frequently caused by mutations in DNA repair genes indicates that reduction in DNA repair gene expression can be a crucial early event in progression to cancer. If DNA repair gene expression is reduced in a somatic tissue by epigenetic repression, this is also likely to be a crucial early event in progression to cancer in that tissue.

Epimutations in DNA repair genes are frequent during progression to cancer

Vogelstein *et al*[15], reviewing evidence from sequencing 3284 tumors and the 294881 mutations found in those cancers, noted that germ-line mutations that give rise to hereditary cancer syndromes are infrequent in sporadic tumors.

More in depth studies of defects in DNA repair genes O-6-methylguanine-DNA methyltransferase (*MGMT*) and *PMS2*, important in progression to GI cancer, are consistent with the observations of Vogelstein *et al*[15]. In the case of *MGMT*, 113 sequential colorectal cancers were evaluated and only four had a missense mutation in the DNA repair gene *MGMT*, while most had reduced *MGMT* expression due to methylation of the *MGMT* promoter region[16]. Other laboratories, quantifying their results, reported that 40% to 90% of colorectal cancers have reduced *MGMT* expression due to methylation of the *MGMT* promoter region[17-21].

In the case of *PMS2*, when 119 colorectal cancers deficient in DNA mismatch repair gene *PMS2* expression were examined, mutation in *PMS2* was present in 6 cases while in 103 cases the pairing partner of *PMS2*, *MLH1* was repressed due to promoter methylation (PMS2 protein is unstable in the absence of MLH1)[22]. In the remaining 10 cases it was likely that epigenetic over-expression of the miRNA, miR-155, which down-regulates MLH1 messenger RNA (mRNA), caused the loss of *PMS2* expression[23].

These findings suggest that, if an early step in progression to sporadic GI cancer is reduction in function of a DNA repair gene, that reduction is likely due to an epigenetic alteration rather than to a mutation in that gene.

DNA DAMAGES ARE VERY FREQUENT AND AN IMPORTANT CAUSE OF CANCER

An average of more than 60000 endogenous DNA damages occur per cell per day in humans (Table 2). These are largely caused by hydrolytic reactions, interactions with reactive metabolites such as lipid peroxidation products, endogenous alkylating agents and reactive carbonyl species, and exposure to reactive oxygen molecules[28].

However, more important still in causing cancer, are DNA damages caused by exogenous agents. Doll *et al*[29] compared cancer rates for 37 specific cancers in the United States to rates for these cancers in countries where there is low incidence for these cancers. The populations for comparison included Norwegians, Nigerians, Japanese, British and Israeli Jews. They concluded that 75%-80% of the cases of cancer in the United States were likely avoidable. They indicated that the avoidable sources of cancer included tobacco, alcohol, diet (especially meat and fat), food additives, occupational exposures (including aromatic amines, benzene, heavy metals, vinyl chloride), pollution, industrial products, medicines and medical procedures, UV light from the sun, exposure to medical X-rays, and infection. Many of these sources of cancer are DNA damaging agents.

One example of diet-related DNA damaging agents likely important in human GI cancer are bile acids. Bernstein *et al*[30] summarized 14 published reports showing that the secondary bile acids deoxycholic acid and lithocholic acid, formed by bacterial action in the colon, cause DNA damage. Bile acids are increased in the colon after the gall bladder releases bile acids into the digestive tract in response to consumption of fatty foods to aid in their digestion. Bile acids in the colon were doubled in the colonic contents of humans in the United States who were on typical diets and then were experimentally fed a high fat diet[31]. Cancer rate comparisons can be made between two similar populations, one with low levels and one with high levels of colonic bile acids. For instance, deoxycholic acid (DOC) in the feces of Native Africans in South Africa is present at 7.30 nmol/g wet weight stool while for African Americans DOC is present at 37.51 nmol/g wet weight stool, a 5.14 fold higher concentration[32]. Native Africans in South Africa have a colon cancer rate

Table 1 Inherited mutations in DNA repair genes that increase the risk of gastrointestinal cancer

DNA repair gene(s)	Repair pathway(s) affected	Cancers with increased risk
BLM	HRR[1]	Leukemia, lymphoma, colon, breast, skin, lung, auditory canal, tongue, esophagus, stomach, tonsil, larynx, uterus[2]
WRN	HRR, NHEJ, long patch BER[3]	Soft tissue sarcoma, colorectal, skin, thyroid, pancreatic[4]
Fanconi's anemia genes *FANC A, B, C, D1, D2, E, F, G, I, J, L, M, N*	HRR and TLS[5]	Leukemia, liver tumors, solid tumors in many areas including esophagus, stomach and colon[6]
MSH2, MSH6, MLH1, PMS2	MMR[7]	Colorectal, endometrial[7]
MUTYH	BER of A mispaired with 8-OHdG[8]	Colon[8]
P53	HRR, BER, NER, NHEJ, MMR[9]	Sarcoma, breast, osteo-sarcoma, brain, adreno-cortical carcinomas[10] and colon and pancreas[11]

HRR: Homologous recombinational repair; NHEJ: Non-homologous end joining; BER: Base excision repair; TLS: Translesion synthesis; MMR: Mismatch repair; DDR: DNA damage response.

Table 2 Endogenous DNA damages/cell/day for humans

DNA damages	Reported rate of occurrence
Oxidative damages	10000[24]
Depurinations	9000[25]
Depyrimidations	696[26]
Single-strand breaks	55000[26]
Double-strand breaks	Approximately 50/cell cycle[27]
O^6-methylguanine	3120[26]
Cytosine deamination	192[26]

of < 1:100000[33] compared to the incidence rate for male African Americans of 72:100000[34], a more than 72-fold difference in rates of colon cancer.

The likely role of bile acids as causative agents in colon cancer is further illustrated by experiments with mice. When mice were fed a diet supplemented with the bile acid deoxycholate (DOC) for 10 mo, raising their colonic level of DOC to that of humans on a high fat diet, 45% to 56% of these mice developed colon cancers, while mice fed the standard diet alone, with 1/10 the level of colonic DOC, developed no colon cancers[35,36].

Another indication that diet is important in colon cancer is observed in populations migrating from low-incidence to high-incidence countries. Cancer rates change rapidly, and within one generation reach the rate in the high-incidence country. This has been observed, for instance, in the colon cancer incidence of migrants from Japan to Hawaii[37].

MANY GENES INVOLVED IN DNA REPAIR

At least 169 enzymes are either directly employed in DNA repair or influence DNA repair processes[38]. Of these, 139 are directly employed in DNA repair processes including base excision repair (BER), nucleotide excision repair (NER), homologous recombinational repair (HRR), non-homologous end joining (NHEJ), mismatch repair (MMR) and direct reversal of lesions (DR). The other 30 enzymes are employed in the DNA damage response (DDR) needed to initiate DNA repair; chromatin structure modification required for repair; reactions needed for the reversible, covalent attachment of ubiquitin and small ubiquitin-like modifier proteins to DDR factors that facilitate DNA repair; or modulation of nucleotide pools.

When the incidence of endogenous and exogenous DNA damages is high, decreases in expression of DNA repair genes or DDR genes lead to a build-up of DNA damage within a cell. These excessive damages provide more opportunities for replication errors and erroneous repair to occur (see mechanisms below) and cause higher rates of mutation and epimutation. Higher numbers of mutations and epimutations increase the chance of including selectively advantageous driver mutations and epimutations that, in turn, promote progression to cancer.

DNA DAMAGES GIVE RISE TO MUTATIONS AND EPIGENETIC ALTERATIONS

Translesion synthesis (TLS) past a single-stranded DNA damage introduces mutations.

Single-strand DNA damages are the most frequent endogenous DNA damages (Table 2). TLS is a DNA damage tolerance process that allows the DNA replication machinery to replicate past single-strand DNA lesions in the template strand. This permits replication to be completed, rather than blocked (which may kill the cell or cause a translocation or other chromosomal aberration)[39].

Humans have four translesion polymerases in the Y family of polymerases [REV1, Pol κ (kappa), Pol η (eta), and Pol ι (iota)] and one in the B family of polymerases [Pol ζ (zeta)][39]. The temporary tolerance of DNA damage during chromosome replication may allow DNA repair processes to remove the damage later[40], and avoid immediate genome instability[41]. However, translesion synthesis is less accurate than the replicative polymerases δ (delta) and ε (epsilon) and tends to introduce mutations[39].

Deficiency in expression of a DNA repair gene can allow excessive DNA damages to accumulate. Some of the excess damages will likely be processed by translesion synthesis, causing increased mutation.

Kunz *et al*[42] summarized numerous experiments

in yeast, in which forward mutations were measured (by sequence analyses of a few selected genes) in cells carrying either wild-type alleles or one of 11 inactivated DNA repair genes. Their results indicated that DNA repair deficient cells accumulate excess DNA damages that then give rise to mutations after error-prone translesion synthesis. The 11 inactivated DNA repair genes were distributed among MMR, *NER*, *BER* and *HRR* genes. Deficiencies in DNA repair increased mutation frequencies by factors between 2- and 130-fold, but most often by double digit-fold increases. Overall, the authors concluded that 60% or more of spontaneous single base pair substitutions and deletions are likely caused by translesion synthesis.

Stuart *et al*[43] examined spontaneous mutation frequencies in a *lacI* transgene (in a Big Blue mutation assay[44]) in either replicating tissues or in largely non-replicating tissues of mice. If most mutations occur during translesion synthesis, then non-replicating brain tissue, which has little or no synthesis once maturity is reached, would have little or no further mutation accumulation. In mouse brain, after 6 mo of age, there was no increase in mutation frequency, even at 25 mo of age. In bladders of mice, with replicating tissues, mutation frequency increased with age, almost tripling between ages of 1.5 mo and 12 mo of age. The authors concluded that the age related increases in spontaneous mutation frequencies reflect endogenous DNA damages that subsequently gave rise to mutations following DNA replication. This indicates that translesion synthesis is a major source of mutation in mouse replicating tissues.

Mutations are frequently caused by error-prone repair of double-strand breaks

While only a minority of endogenous DNA damages in the average cell are double-strand breaks (Table 2), this type of lesion appears to contribute substantially to the mutation rate as well. As indicated by Vilenchik and Knudson[27], the doubling dose for ionizing radiation (IR) induced double-strand breaks is similar to the doubling dose for mutation and induction of carcinomas by IR. Thus, double-strand breaks likely lead frequently to mutations.

As described by Bindra *et al*[45], non-homologous end-joining (NHEJ) and HRR comprise the two major pathways by which double-strand breaks (DSBs) are repaired in cells. NHEJ processes and re-ligates the exposed DNA termini of DSBs without the use of significant homology, whereas HRR uses homologous DNA sequences as a template for repair. HRR predominates in S-phase cells, when a sister chromatid is available as a template for repair, and is a high-fidelity process. NHEJ is thought to be active throughout the cell cycle, and it is more error-prone than HRR. NHEJ repair comprises both canonical NHEJ and non-canonical pathways. The former pathway results in minimal processing of the DSB during repair, whereas the latter pathway, with or without the use of sequence microhomology for re-ligation, typically results in larger insertions or deletions. Mutagenic NHEJ repair is a robust process, yielding percentages of mutated sites at the position of a DSB ranging from 20% to 60%.

As pointed out by Vilenchik *et al*[27], about 1% of single-strand DNA damages escape repair and are not bypassed, and some of these become converted to double-strand breaks. This may contribute to the impact of double-strand breaks in causing mutations and carcinogenesis.

Epigenetic alterations occur due to DNA damage

Epigenetic alterations can arise due to incomplete repair of DNA double-strand breaks. As an example, O'Hagan *et al*[46] used a cell line stably transfected with a plasmid containing a consensus *I-SceI* cut site inserted into a copy of the *E-cad* promoter. This promoter contained a CpG island. O'Hagan *et al*[46] induced a defined double-strand break in the E-cadherin CpG island, and the CpG island was not currently hypermethylated. As the repair of the break began, they observed that key proteins involved in establishing and maintaining transcriptional repression were recruited to the site of damage, to allow repair of the break. Most cells examined after the DNA break was repaired showed that DNA repair occurred faithfully, with the promoter not hypermethylated and the silencing factors removed. However, a small percentage of the cells retained heritable silencing. In these cells the chromatin around the break site was enriched for most of the silencing chromatin proteins and histone marks, and the region had increased DNA methylation in the CpG island of the promoter. Thus, repair of a DNA break can occasionally cause heritable silencing of a CpG island-containing promoter. Such CpG island methylation is frequently associated with tight gene silencing in cancer.

Morano *et al*[47] also showed that epigenetic alterations can arise as a consequence of DNA damage. They studied a system in which recombination between partial duplications of a chromosomal green fluorescent protein (*GFP*) gene is initiated by a DSB in one copy. Two cell types were generated after recombination: clones expressing high levels of GFP and clones expressing low levels of GFP, referred to as H and L clones, respectively. Relative to the parental gene, the repaired *GFP* gene was hypomethylated in H clones and hypermethylated in L clones. The altered methylation pattern was largely restricted to a segment 3' to the DSB. Although it is 2000 base pairs distant from the strong cytomegalovirus promoter that drives GFP expression, hypermethylation of this tract significantly reduced transcription. The ratio of L (hypermethylated) to H (hypomethylated) clones was 1:2 or 1:4, depending on the insertion site of the GFP reporter. These experiments were performed in mouse embryonic (ES) or human cancer (Hela) cells. HRR-induced methylation depended on DNA

methyltransferase I. These data, taken together, argue for a cause-effect relationship between double-strand DNA damage-repair and altered DNA methylation.

The main function of the proteins in the BER pathway is to repair DNA single-strand breaks and deamination, oxidation, and alkylation-induced DNA base damage. In addition, Li *et al*[48] reviewed studies indicating that one or more BER proteins also participate(s) in epigenetic alterations involving DNA methylation, demethylation or reactions coupled to histone modification. Franchini *et al*[49] also showed that DNA demethylation can be mediated by BER and other DNA repair pathways requiring processive DNA polymerases. Another form of epigenetic silencing also appears to occur during DNA repair. PARP1 [poly(ADP)-ribose polymerase 1] and its product poly(ADP)-ribose (PAR) accumulate at sites of DNA damage as intermediates of a DNA repair process[50]. This directs recruitment and activation of the chromatin remodeling protein ALC1, which can cause nucleosome remodeling[51]. Nucleosome remodeling, in one case, has been found to cause epigenetic silencing of DNA repair gene *MLH1*[52]. These reports, overall, indicate that DNA damages needing repair can cause epigenetic alterations by a number of different mechanisms.

Other causes of epigenetic alterations

Heavy metals and other environmental chemicals cause many epigenetic alterations, including DNA methylation, histone modifications and miRNA alterations[53]. DNA damage itself causes programmed changes in non-coding RNAs, and a large number of miRNAs are transcriptionally induced upon DNA damage[54]. However, it is not clear what proportion of these alterations are reversed or are retained as epimutations after the external sources of damage are removed upon repair of the DNA damages[55].

Mutations in isocitrate dehydrogenase 1 (IDH1) and *2* (IDH2) are frequent in several types of cancer and they can cause epigenetic alterations. As reviewed by Wang *et al*[56], *IDH1* and *IDH2* mutations represent the most frequently mutated metabolic genes in human cancer. These mutations occur in more than 75% of low grade gliomas and secondary glioblastoma multiforme, 20% of acute myeloid leukemias, 56% of chondrosarcomas, over 80% of Ollier disease and Maffucci syndrome, and 10% of melanomas. *IDH1* is also mutated in 13% of inflammatory bowel disease-associated intestinal adenocarcinoma with low-grade tubuloglandular histology but not in sporadic intestinal adenocarcinoma[57]. The *IDH1* and *IDH2* mutations that give rise to epimutations usually occur in the hotspot codons Arg132 of *IDH1*, or the analogous codon Arg172 of *IDH2*. These mutations allow accumulation of the metabolic intermediate 2-hydroxyglutarate (2-HG), and 2-HG inhibits the activity of alpha ketoglutarate (α-KG) dependent dioxygenases, including α-KG-dependent histone demethylases and the TET family of 5-methylcytosine hydroxylases.

Wang *et al*[56] found that histone H3K79 dimethylation levels were significantly elevated in cholangiocarcinoma samples that harbored *IDH1* or *IDH2* mutations (80.8%) compared to tumors with wild-type *IDH1* and *IDH2* (45.0%).

In addition, they surveyed over 462000 CpG sites in CpG islands, CpG shores and intragenic regions, and found that 2309 genes had significantly increased methylation in the presence of *IDH1* or *IDH2* mutations. In particular, Sanson *et al*[58] found that methylation of the DNA repair gene *MGMT* was associated with *IDH1* mutation, since 81.3% of *IDH1*-mutated gliomas were *MGMT* methylated compared with 58.3% methylated in *IDH1* non-mutated tumors.

DNA REPAIR GENES WITH EPIGENETICALLY REDUCED EXPRESSION ARE LIKELY PASSENGERS IN A SPREADING FIELD DEFECT

A DNA repair gene that is epigenetically silenced or whose expression is reduced would not likely confer any selective advantage upon a stem cell. However, reduced or absent expression of a DNA repair gene would cause increased rates of mutation, and one or more of the mutated genes may cause the cell to have a strong selective advantage. The expression-deficient DNA repair gene could then be carried along as a selectively neutral or only slightly deleterious passenger (hitch-hiker) gene when there is selective expansion of the mutated stem cell. The continued presence of a DNA repair gene that is epigenetically silenced or has reduced expression would continue to generate further mutations and epigenetic alterations.

The spread of a clone of cells with a selective advantage, but carrying along a gene with epigenetically reduced expression of a DNA repair protein would be expected to generate a field defect, from which smaller clones with still further selective advantages would arise. This is consistent with the finding of field defects in colonic resections, that have both a cancer and multiple small polyps, such as the one shown in Figure 1.

For any particular type of GI cancer, an epigenetic reduction in expression of a specific DNA repair gene may be common. In cases where a specific epigenetic reduction of expression of a DNA repair gene occurs in a cancer, it is also likely to be evident in the field defect surrounding the cancer (Table 3). The lower frequency in the surrounding field defect that is usually found (Table 3) likely reflects the process whereby the expanding clone laterally displaces the more normal epithelium. This displacement may be only partial. Thus, areas with the DNA repair deficiency would be present at a lower frequency in the field defect than in the cancer. In the cancer, the cells carrying the DNA repair deficiency are members of a founding clone. Thus, in the cancer, the DNA repair defect, along

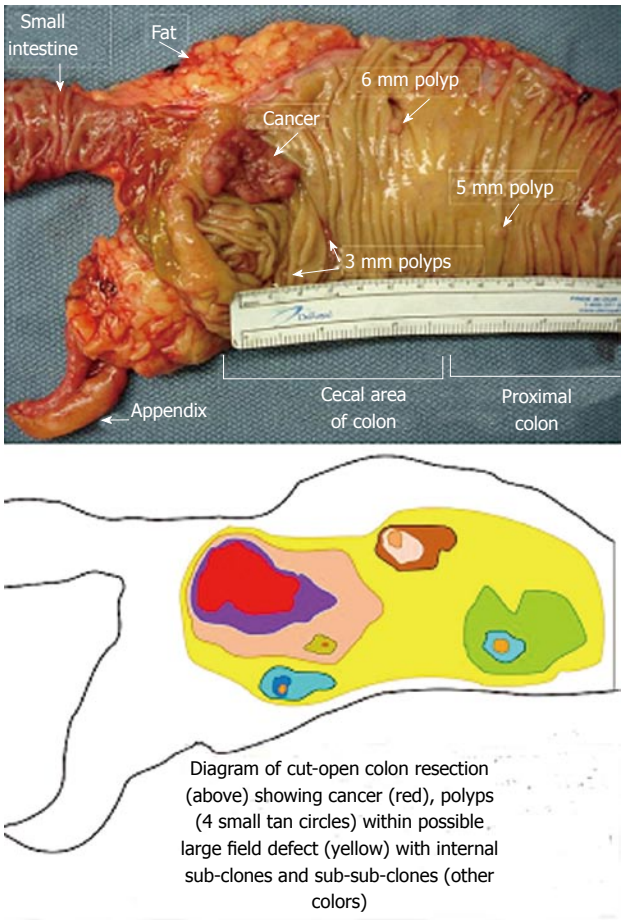

Figure 1 Cut open gross specimen of proximal human colon showing multiple tumors[59].

with other accumulated mutations and epigenetic alterations, would be seen at a relatively higher frequency than in the surrounding field defect.

DECREASED EXPRESSION OF MULTIPLE DNA REPAIR GENES IN GI CANCERS

The protein expressions of three DNA repair genes within a 20 cm colon resection were evaluated at six different locations within the resection (Figure 2)[62]. One of the DNA repair proteins, KU86, was only deficient infrequently, with the deficiencies occurring in small patches (up to three crypts). These KU86 defects are not likely important in progression to colon cancer. However, two of the evaluated DNA repair proteins, ERCC1 and PMS2, were often deficient in patches of tens to hundreds of adjacent crypts at each of the locations evaluated (see Nguyen *et al*[68] at minutes 18 to 24 of a 28 min video of crypts immunostained for ERCC1 or PMS2).

Overall, ERCC1 (NER) was deficient in 100% of 49 colon cancers evaluated, and in an average of 40% of the crypts within 10 cm on either side of the cancer. PMS2 (MMR) was deficient in 88% of the 49 cancers and in an average of 50% of the crypts within 10 cm of the cancer. As reported by Facista *et al*[62], the pattern

Table 3 Epigenetic deficiency of DNA repair genes in gastrointestinal cancers and field defects

Cancer	Gene	Frequency in cancer	Frequency in adjacent field defect
Colorectal[17]	*MGMT*	46%	34%
Colorectal[19]	*MGMT*	47%	11%
Colorectal[60]	*MGMT* with *MSI*	70%	60%
Colorectal[19]	*MSH2*	13%	5%
Colorectal[61]	*MBD4*	Frequent	Frequent
Colorectal[62]	*ERCC1*	100%	40%
Colorectal[62]	*PMS2*	88%	50%
Colorectal[62]	*XPF*	55%	40%
Colorectal[63]	*WRN*	29%	13%
Stomach[64]	*MGMT*	88%	78%
Stomach[65]	*MLH1*	73%	20%
Esophagus[66]	MLH1	77%-100%	23%-79%

MSI: Microsatellite instability.

of expression of ERCC1 in the crypts within 10 cm of a colon cancer indicated that when the ERCC1 protein was deficient, this deficiency was due to an epigenetic reduction in expression of the *ERCC1* gene. When the PMS2 protein is deficient, it is usually due to the epigenetic repression of its pairing partner, MLH1, and the instabilty of PMS2 in the absence of MLH1[22]. In the study of Facista *et al*[62], ERCC1 and PMS2 were also deficient in all 10 tubulovillous adenomas evaluated (precursors to colonic adenocarcinomas). Thus ERCC1 and PMS2 are deficient at early times (in the field defect), at intermediate times (in tubulovillus polyps), and at late times (within the cancer) during progression to colon cancer. Another DNA repair protein, XPF, was deficient in 55% of the cancers, as well[62]. The majority of cancers were simultaneously deficient for ERCC1, PMS2 and XPF.

Deficiencies in multiple DNA repair genes were also observed in gastric cancers. Kitajima *et al*[69] evaluated MGMT (direct reversal repair), MLH1 (MMR) and MSH2 (MMR) and found that synchronous losses of MGMT and MLH1 increase during progression and stage of differentiated-type cancers. In un-differentiated-type gastric cancers, the frequency of MGMT deficiency increased from early to late stages of the cancer, while frequencies of MLH1 and MSH2 deficiencies were between 48% and 74% at both early and late stages. Thus, in un-differentiated-type gastric cancers, MLH1 or MSH2 deficiency, if it is present, is an early step, while MGMT deficiency is often a later step in progression of this cancer.

Farkas *et al*[70] evaluated 160 genes in 12 paired colorectal tumors and adjacent histologically normal mucosal tissues for differential promoter methylation. They found aberrant methylation in 23 genes, including six DNA repair genes. These DNA repair genes (with DNA repair pathways indicated) were *NEIL1* (BER), *NEIL3* (BER), *DCLRE1C* (NHEJ), *NHEJ1* (NHEJ), *GTF2H5* (NER), and *CCNH* (NER).

Lynam-Lennon *et al*[71] found that miR-31 is over-

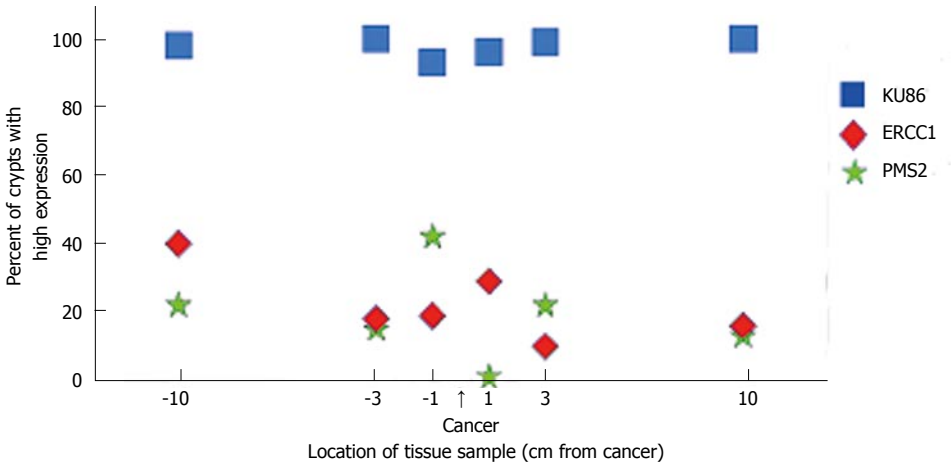

Figure 2 Expression of three DNA repair proteins, KU86, ERCC1 and PMS2, at locations sampled along the 20 cm length of a colon resection that had a cancer at the indicated location[67].

expressed in 47% of esophageal cancers and examined the consequences of over-expression of miR-31 in these cancers. Using a cell line, they first tested the effect of over-expression of miR-31 on the expression of 84 DNA repair genes. They found that 11 DNA repair genes were repressed by over-expression of miR-31. They then evaluated the expression of the five most altered DNA repair genes in 10 esophageal cancers that had high expression of miR-31 and low resistance to radiation treatment (likely low levels of DNA repair). These 10 cancers showed significantly reduced mRNA levels of DNA repair genes *PARP1, SMUG1, MLH1* and *MMS19*. Asangani *et al*[72] showed that miR-31 is an epigenetically regulated microRNA. This microRNA is encoded in an intron of *MIR31HG* (*miR-31* host gene). The transcriptional regulatory region of *MIR31HG* is enriched for histone 3 that could be acetylated on lysine (K) 27 (this is designated H3K27Ac), and H3K27Ac causes an epigentic "mark" that is associated with transcriptionally active genes. If, instead, this histone 3 has triple methylation on lysine 27 (H3K27me3), this causes gene silencing. The regulatory region of *MIR31HG* also has 77 CpG islands surrounding the transcription start site. These observations indicate that miR-31 transcription could be up-regulated by H3K27Ac or silenced by CpG island methylation or by histone H3K27me3. It appears that DNA repair genes *PARP1* (BER and HRR), *SMUG1* (BER), *MLH1* (MMR) and *MMS19* (NER) are epigenetically repressed by over-expressed miR-31 in esophageal cancers.

Based on the examples above, decreased expression of multiple DNA repair genes likely occurs often in GI neoplasia.

EFFECTS LIKELY DUE TO DNA REPAIR DEFECTS

Regression of early lesions

If DNA repair defects are present early in progression to cancer, this should result in increased mutation frequency in those neoplastic lesions. Most new mutations are expected to be deleterious to the cells in which they arise, and thus would cause negative selection of those cells. This expectation is consistent with the observations of Hofstad *et al*[73] who showed that when colonic polyps were identified during a colonoscopy and followed but not removed, between 11% and 46% of polyps smaller than 5 mm diameter were not detectable in the succeeding one to three years. For polyps between 5 and 9 mm in diameter, between 4% and 24% became undetectable in the succeeding one to three years. Of the remaining 68 polyps that were followed for three years, 35% decreased in diameter, 25% remained the same size and 40% increased in diameter. Similarly, Stryker *et al*[74] followed 226 patients with colonic polyps that were $\geqslant$ 1 cm in size for an average of 5.7 years (though some patients were followed for as long as 19 years). Stryker *et al*[74] found that 37% of polyps $\geqslant$ 1 cm enlarged (at least doubled in volume) during the study while 4% of the polyps that had been observed at least twice, previously, were later not found. The risk of these polyps $\geqslant$ 1 cm producing an invasive carcinoma within 20 years was 24%. The data of Hofstad *et al*[73] and Stryker *et al*[74] are also consistent with statistics showing more frequent occurrence of adenomas during colonoscopy and autopsy compared to the frequency of colon cancer, indicating there must be a significant regression rate for adenomas[75].

Subclones in cancers

When infrequent positively selected mutations arise in a cell, this can provide the cell with a competitive advantage that promotes its preferential clonal proliferation, leading to cancer. The continued presence of epigenetically repressed DNA repair genes, carried along as passengers in the development of cancers, also predicts that cancers will contain heterogeneous genotypes (multiple subclones). For instance, as a test for the presence of subclones, in one primary

renal carcinoma with multiple metastases, 101 non-synonymous point mutations and 32 indels (insertions and deletions) were identified[76]. Five mutations were not validated and excluded from the study. Of the remaining 128 mutations, 40 were "ubiquitous" and present in each region of the tumor sampled. There were 59 "shared" mutations, present in several but not all regions, and 29 "private" mutations, unique to a specific region evaluated. The authors constructed a phylogenetic tree and concluded that the evolution in the tumor and its metastases was branching, and not linear.

A deficiency of DNA repair would likely produce genetic clonal diversity, through generation and selection for new mutational variants. In a study by Maley *et al*[77], 268 patients with Barrett's esophagus were followed for an average of 4.4 years during which 37 esophageal adenocarcinomas (EACs) developed. Genetic clonal diversity within Barrett's esophagus proved to be a better predictor of EAC than the presence of specific mutations in genes associated with EAC, such as mutation in *P53*. This finding suggests that DNA repair deficiency is of primary importance in progression to cancer.

EPIGENETIC REPRESSION OF DNA REPAIR GENES, DUE TO ALTERATIONS IN CPG ISLAND METHYLATION IN GI CANCERS

Table 4 gives examples of reports of DNA repair genes repressed by CpG island hypermethylation (or with increased expression due to CpG hypomethylation, which may cause unbalanced repair processes) in GI cancers (this is only a partial list). Nine different DNA repair genes (all listed among the 169 DNA repair and *DDR* genes previously identified[38]) were often hyper- (or sometimes hypo-) methylated in one or more GI cancer. Such alterations in methylation of promoter regions of DNA repair genes can cause deficient repair of DNA damages. Thus, hyper- (or hypo-) methylations of DNA repair genes are frequently important factors responsible for lack of appropriate repair of DNA damages. Faulty DNA repair leads to increased mutation and epigenetic alteration, central to progression to cancer.

DNA REPAIR GENE EXPRESSION MAY BE REPRESSED BY MULTIPLE PROCESSES

A number of the DNA repair genes with reduced expression due to CpG island hypermethylation are also epigenetically repressed by other means. Many protein coding genes are repressed by microRNAs. MicroRNAs (miRNAs) are small noncoding endogenously produced RNAs that play key roles in controlling the expression

Table 4 CpG island hyper- (and hypo-) methylation of DNA repair genes in cancers

Cancer	Gene	Frequency of promoter hyper- (or hypo-) methylation in cancer
Colorectal	*LIG4*	82%[78]
	MGMT	40%-90%[17-21]
	ERCC1	38%[79]
	WRN	29%-38%[63,80]
	MLH1	9%-10%[22,81]
	FEN1	Frequent (hypo-)[82]
	MBD4	Frequent (hyper-)[61]
Esophageal	*MGMT*	23%-79%[65,83,84]
	MLH1	43%[82], 64%[85]
	MSH2	29%[83], 75%[84]
Stomach	*MGMT*	88%[60]
	MLH1	73%[64]
	WRN	24%-25%[80,86]
	FEN1	Frequent (hypo-)[82]
Gastric lymphoma	*ATM*	11%[87]

of many cellular proteins. Once they are recruited and incorporated into a ribonucleoprotein complex, they can target specific messenger RNAs (mRNAs) in a miRNA sequence-dependent process and interfere with the translation into proteins of the targeted mRNAs *via* several mechanisms (see detailed review by Lages *et al*[88]).

As discussed above, when mismatch DNA repair protein PMS2 is deficient in colorectal cancer, this may be due to hypermethylation of its pairing partner MLH1, or due to over-expression of the miRNA miR-155 which targets the *MLH1* gene for repression.

While only 38% of cancers have CpG island methylation of the *ERCC1* promoter (Table 4), Facista *et al*[62] found that 100% of colon cancers have significantly reduced levels of ERRC1 protein expression. In the 49 cancers examined, ERCC1 protein expression varied from 0% to 45% (with a median value of 28%) of the level of ERCC1 expression of neoplasm-free individuals. It is likely that *ERCC1* can be repressed by more than one mechanism. A second mechanism of repression of *ERCC1* may be due to the combined effects of epigenetically deficient miRNA let-7a and resulting over-expression of HMGA2 protein, which then represses *ERCC1,* as discussed below.

As indicated by Motoyama *et al*[89], the let-7a miRNA normally represses the *HMGA2* gene, and in normal adult tissues, almost no HMGA2 protein is present. In breast cancers, for instance, the promoter region controlling let-7a-3/let-7b miRNA is frequently repressed by hypermethylation[90]. Reduction or absence of let-7a miRNA allows high expression of the HMGA2 protein. Regulation of gene expression by HMGA2 is achieved by binding to AT-rich regions in the DNA and/or direct interaction with several transcription factors[91].

HMGA2 targets and modifies the chromatin architecture at the *ERCC1* gene, reducing its expression[92]. As shown by Mayr *et al*[93], using an artificial construct,

the lack of let-7a miRNA repression of HMGA2 could occur through translocation of *HMGA2*, disrupting the 3' UTR of *HMGA2* which is the target of let-7a miRNA, and this can lead to an oncogenic transformation. However, the promoter controlling let-7a miRNA also can be strongly regulated by hypermethylation in intact cells. When human lung cells are exposed to cigarette smoke condensation, the promoter region controlling let-7a becomes highly hypermethylated[94]. It is likely that hypermethylation of the promoter for let-7a miRNA reduces its expression. This allows hyperexpression of *HMGA2*. Hyperexpression of *HMGA2* can then reduce expression of *ERCC1.* The combined effects of reduced let-7a miRNA and hyperexpressed *HMGA2* or other possible epigenetic mechanism(s) may cause the reduced protein expression of ERCC1 in colorectal cancers in addition to the 38% of colorectal cancers in which the *ERCC1* gene is directly hypermethylated.

DNA REPAIR PROTEINS AND MIRNAS

A review by Wouters *et al*[95] lists 74 DNA repair genes that are potentially targeted by miRNAs, and two additional reviews[96,97] list, combined, 30 miRNAs known to target DNA repair genes. The review by Wouters *et al*[95] used "in silico" computer programs (Targetscan and Mirbase) to identify likely miRNAs that could target their 74 DNA repair genes of interest, and, for each of these genes, indicated between 1 and 19 "conserved" miRNAs that were predicted to repress those genes. They define "conserved" miRNAs as miRNAs found in at least five mammalian species. However, about half of the miRNAs they found "in silico" were inducible by UV irradiation, and may have been controlled by transcriptional regulation and not by an epigenetic mechanism. Tessitore *et al*[96] and Vincent *et al*[97] each list about 20 miRNAs that are altered in cancers and which control expression of DNA repair genes. However, they did not indicate how these miRNAs are deregulated.

Deregulation of miRNA expression in cancers has been found to occur by epigenetic as well as non-epigenetic mechanisms[88,98]. One non-epigenetic mechanism includes alterations in genomic miRNA copy numbers and location. Some of these are deletions that include the miRNA clusters *15a/16-1* or *let-7g/mir-135-1,* or else amplification or translocation of the *mir-17-92* cluster. In some cancers miRNAs were deregulated because of defects in the biogenesis mechanism (the process of creating miRNAs, which has a number of steps). Some cancers have deregulated miRNAs due to single nucleotide polymorphisms (SNPs) in the genes coding for the miRNAs, or SNPs in the target gene area to which the miRNA is targeted. Some miRNAs, that target DNA repair genes, are regulated by oncogenes. For instance ATM is down-regulated by miR-421, but miR-421 is regulated by N-Myc[99]. Thus, not all instances of deregulation of DNA repair genes or DDR genes by miRNAs are due to epigenetic alterations affecting expression of the miRNAs.

EPIGENETIC REPRESSION OF DNA REPAIR GENES DUE TO ALTERATIONS OF METHYLATION OF PROMOTERS OF MIRNAS IN VARIOUS CANCERS

Table 5 lists nine miRNAs that have three characteristics: (1) their expression is epigenetically controlled by the methylation level of the promoter regions coding for the miRNAs; (2) they control expression of DNA repair genes; and (3) their level of expression was frequently epigenetically altered in one or more types of GI cancer. This list is not exhaustive. Many of the 30 miRNAs listed by Tessitore *et al*[96] or Vincent *et al*[97] might also meet these criteria upon further examination. Four of the miRNAs on this list are not noted by Tessitore *et al*[96] or Vincent *et al*[97]. Most of the studies of these epigenetically controlled miRNAs have not noted the frequencies with which their alterations occur in cancers. Thus, these studies are somewhat less systematic than those detailing methylation of DNA repair genes in Table 4. However, the nine epigenetically controlled miRNAs listed in Table 5 can repress the 16 DNA repair genes listed in Table 5 and these genes are repressed in various GI cancers.

WHOLE GENOME SEQUENCING INDICATES A HIGH LEVEL OF MUTAGENESIS IN GI CANCERS

Almost 3000 pairs of tumor/normal tissues were analyzed for mutations by whole exome sequencing (sequencing the protein coding parts of whole genomes) and more than a hundred pairs of tumor/normal tissues were analyzed for mutations by whole genome sequencing by Lawrence *et al*[120]. Median mutation frequencies for 27 different types of cancer were found to vary by 1000-fold. When there was a particular median mutation frequency for a type of cancer, the scatter of values (in individual cancers) for that type of cancer, above and below that median value, sometimes also varied by as much as 1000-fold. Some mutation frequencies in GI cancers, given as numerical values of median numbers of mutations per megabase in a review of the literature by Tuna *et al*[121], and recent values for esophageal cancers by Weaver *et al*[122], are shown in Table 6. The values were also converted to mutation frequency per whole diploid genome.

The mutation frequency in the whole genome [not just the exome (protein coding regions)] between generations for humans (parent to child) is about 30-70 new mutations per generation[123-125]. For protein

Table 5 Epigenetic ↑ or ↓ miRNAs, altered in cancers, targeting DNA repair genes

Specific miRNA	DNA repair gene targets	Cancers affected (frequency if measured)	References indicating epigenetic control of miRNA	References indicating target gene(s) of miRNAs	References indicating cancer type(s) affected
miR-103 miR-107	*RAD51,* *RAD51D*	Osteosarcoma, lung, endometrial, stomach	[100]	[101]	[101]
miR-34c	*UNG*	Gastric (70%) field defect gastric (27%) colon (98%) field defect colon (60%) chronic lymphocytic leukemia (18%) small-cell lung cancer (67%) NSCLC (26%)	[102,104]	[103]	[102,105,106]
miR-31	*PARP1* *MLH1* *SMUG1* *MMS19*	Esophagus (47%) colon	[72]	[21]	[71,107,108]
miR-124	*KU70*	Colon	[109]	[110]	[109]
miR-155	*RAD51* *MLH1* *MSH2* *MSH6*	Breast Colon	[90,111]	[23,112]	[23,90]
let-7a repression increases HMGA2; HMGA2 alters chromatin architecture of and represses ERCC1)	*ERCC1*	(Colon) Anaplastic astrocytoma	[90]	[92,113]	[113]
Let-7b repression increases HMGA1; HMGA1 targets P53	*P53*	Prostate Colon	[90]	[114,115]	[114,115]
miR-182	*BRCA1* *NBN* *RAD17*	Breast Colon	[116]	[117,118]	[107,117,119]

coding regions of the genome in individuals without cancer, Keightley[126] estimated there would be 0.35 mutations per parent to child generation. Whole genome sequencing was also performed in blood cells for a pair of monozygotic (identical twin) 100 years old centenarians[127]. Only 8 somatic differences were found between the twins, though somatic variation occurring in less than 20% of blood cells would be undetected. These findings, as well as the data summarized in Table 6, indicate that cancer cell lineages experience substantially higher mutation rates than non-cancer cell lineages.

EPIGENETICALLY REDUCED EXPRESSION OF DNA REPAIR GENES IN GI CANCERS OCCUR IN DIFFERENT REPAIR PATHWAYS

Figure 3[128] indicates some types of DNA damaging agents that may be encountered by cells in the GI tract, some of the DNA lesions they cause and the pathways used to repair these lesions. Many of the genes active in these pathways are included in Figure 3 and are indicated by their acronyms. The acronyms listed in red represent genes whose expression is frequently reduced due to epigenetic alterations in various types of GI cancers, as discussed above. Such reduced expression could be a substantial source of the genomic instability that is characteristic of these cancers.

THE CENTRAL ROLE OF DNA DAMAGE AND EPIGENETIC DEFECTS IN DNA REPAIR DURING PROGRESSION TO GI CANCER

The central role of DNA damage and epigenetic defects in DNA repair are illustrated in Figure 4[129]. When DNA damage results in epigenetic reduction in expression of one or more DNA repair genes, the resulting DNA repair deficiency can allow DNA damage to accumulate at a much increased rate. As indicated in Figure 3, at least 18 DNA repair genes that are frequently epigenetically deficient in one or more GI cancers have been identified. These epigenetic defects in DNA repair are often found to be present in field defects from which the cancers arose, so that such epigenetic reductions in DNA repair are likely early events in progression to cancer. A large increase in unrepaired DNA damage, due to an epigenetic reduction in DNA repair, can then lead to the large increase in mutation frequencies found in GI cancers (Table 6).

An epigenetic reduction of DNA repair may be the key early event that accelerates progression to cancer.

SELECTIVE TUMOR KILLING

DNA-damaging agents have a long history of use in cancer chemotherapy. As pointed out by Cheung-Ong *et al*[130], and indicated in the text earlier in this article,

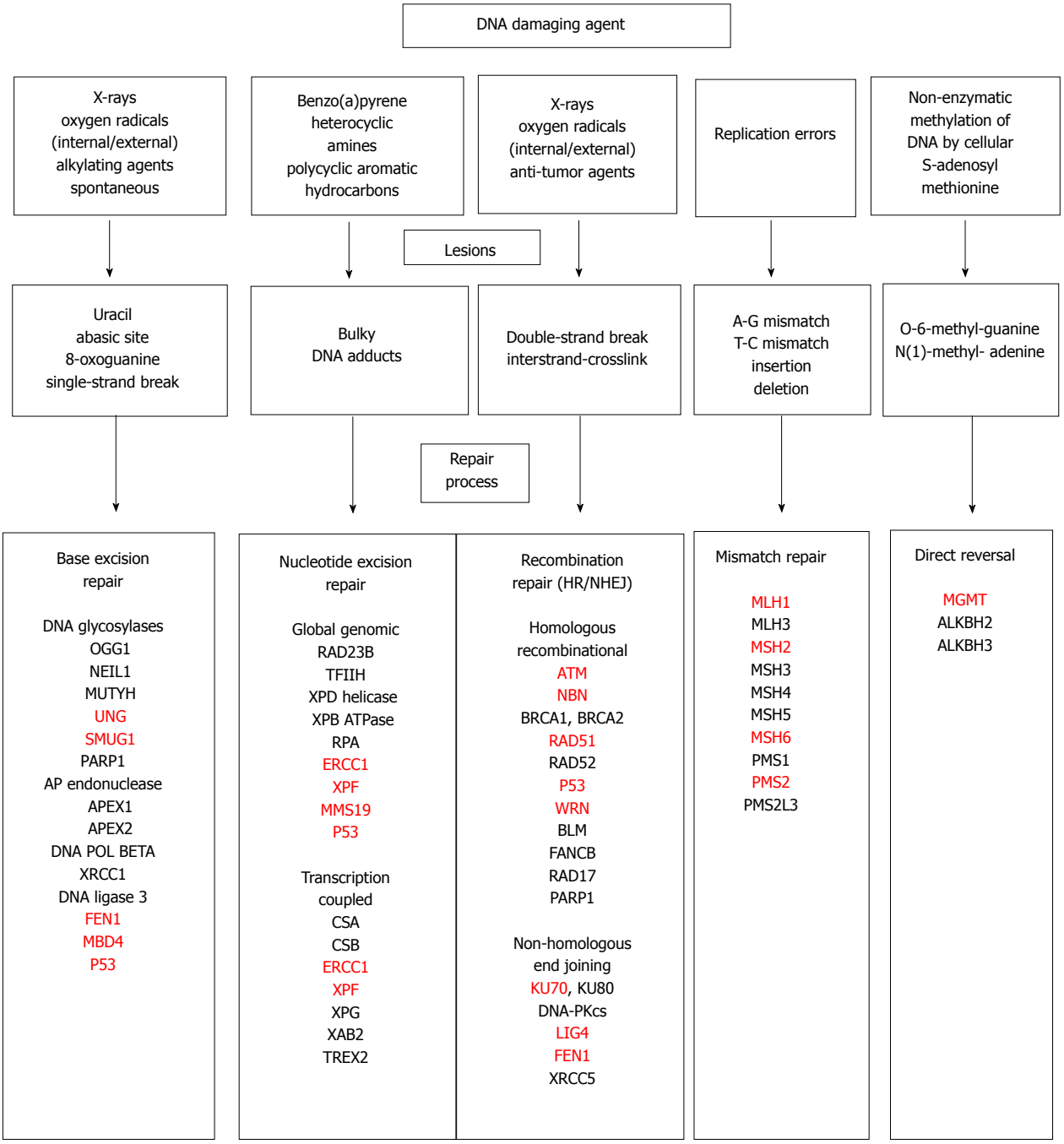

Figure 3 DNA damaging agents, the lesions they produce and the repair pathways that deal with the DNA damages, including acronyms for many of the genes in each of the pathways. Acronyms in red represent genes indicated in the text that have altered (usually reduced) expression due to an epigenetic alteration in one or more types of gastrointestinal cancer[128].

cancer cells are typically deficient in DNA damage-sensing/repair capabilities. That makes them more susceptible to DNA damage than normal cells. As Cheung-Ong *et al*[130] describe, both the earliest as well as the most frequent current cancer chemotherapeutic agents are DNA damaging agents.

A recently developing strategy for more effective and selective treatment of cancer is to inhibit one of the tumor's remaining DDR or DNA repair pathways. This can hyper-sensitize a tumor to radiation or chemotherapeutic agents, compared to the sensitivity of a tumor treated with a DNA damaging agent alone. This strategy is called synthetic lethality.

An early effort to implement synthetic lethality was the successful trial of Fong *et al*[131], in which a PARP inhibitor was given to germ-line mutated *BRCA* carriers. In this case, 12 of 19 (63%) of these patients in a Phase I trial had a clinical benefit from treatment with the PARP inhibitor olaparib alone, with no other chemotherapy. The patients in this Phase I trial had tumors that had been refractory to the 1 - ⩾ 4 therapies that had been tried previously. As noted by O'Sullivan *et al*[132], the BRCA proteins are active in the HRR pathway, and PARP is largely active in BER, though

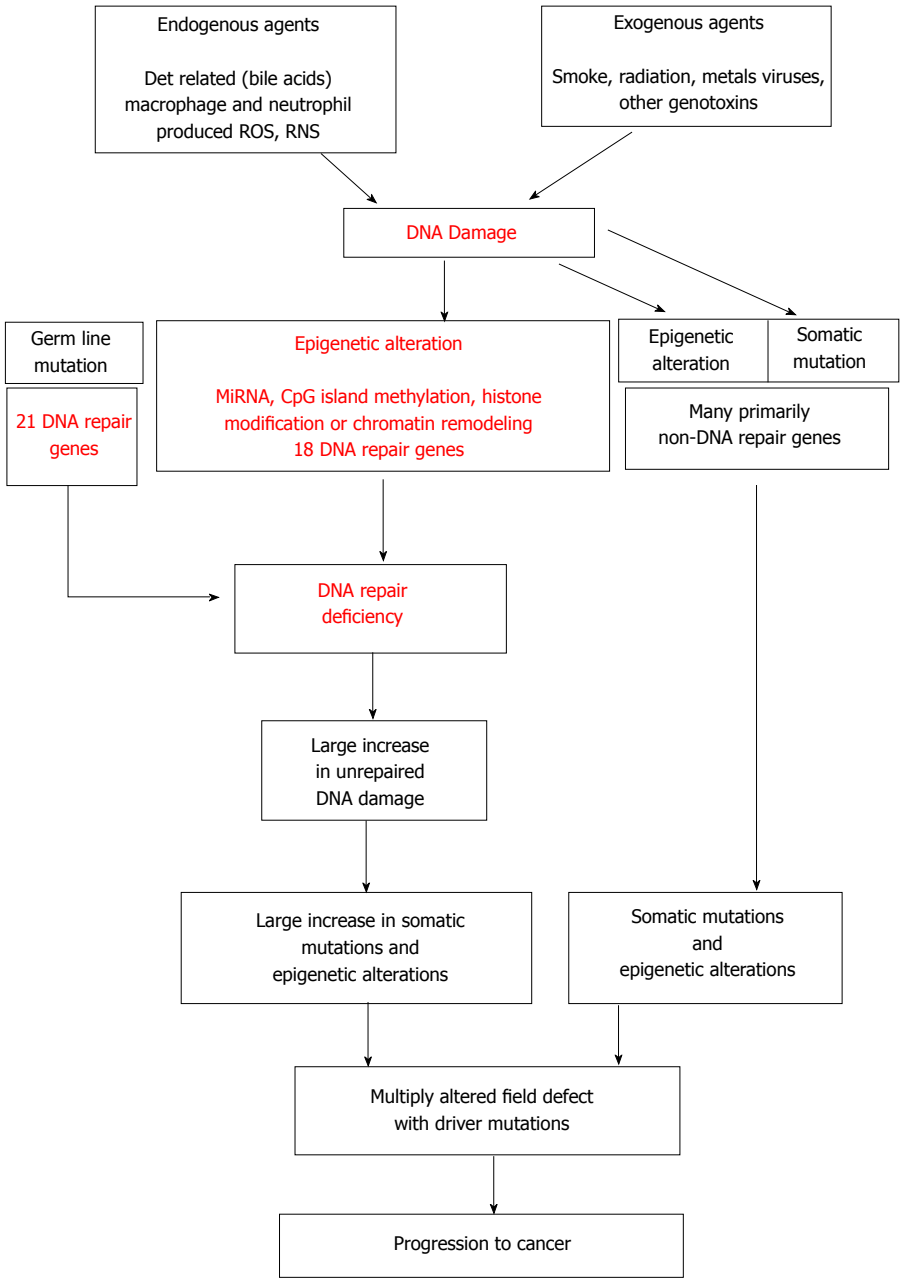

Figure 4 The central role of DNA damage and epigenetic alteration in DNA repair genes in gastrointestinal carcinogenesis[129].

it is also important in HRR. O'Sullivan *et al*[132] indicated that PARP inhibition appears to have synthetic lethality for both BRCA mutation-associated and "BRCA-like" solid tumors. As reviewed by O'Sullivan *et al*[132], PARP inhibitors are currently being evaluated in Phase Ⅰ and Phase Ⅱ trials of many different cancers, including GI cancers in pancreas, liver, colorectum, stomach and esophagus. They summarize some early quantitative results (in the range of 14% to 23% tumor regression or delayed progression) in pancreatic and colorectal cancers. McLornan *et al*[133] summarize positive results (tumor regression or delayed progression), often in the range of about 40% to 50%, with PARP inhibitors used in treatment of advanced solid tumors in other Phase Ⅰ and Ⅱ trials, including one on recurrent or metastatic gastric cancer.

Hosoya *et al*[134] listed a large number of synthetic lethality Phase Ⅰ and Phase Ⅱ trials that included not only PARP inhibitors but also inhibitors of DDR elements CHK1 and CHK2 and inhibitors of DNA repair elements DNA-PK and APE1. In addition they discuss interesting pre-clinical, potentially useful, synthetic lethal experiments with inhibitors of ATM/ATR and the MRN complex, DNA ligases, RAD51, RAD52 and histone deactylases.

Clinical applications of synthetic lethality are just beginning, as Phase Ⅰ and Ⅱ trials, but appear to be a new and potentially effective avenue for cancer therapy. How synthetic lethality may relate to epigenetically repressed DNA repair genes is currently unclear. The

Table 6 Median mutation frequencies and ranges

Parent/child per generation or cancer type	Mutation frequency per million bases	Mutation frequency per diploid genome
Parent/child per generation	0.00000023	30-70
Colorectal carcinoma	Approximately 5	Approximately 30000
MSS colon cancer	2.8	16800
MSI colon cancer (mismatch DNA repair deficient)	47	282000
Hepatocellular carcinoma	4.2	25200
Esophageal carcinoma (single nucleotide variants)	2.8 Range 0.7-9.3	16994 Range 4516-56528
Esophageal carcinoma (small insertions and deletions)		994 Range 262-3573

MSS: Microsatellite stable; MSI: Microsatellite instable.

epigenetic repression of DNA repair genes appears to be important for progression for many types of cancer, for cancer susceptibility to DNA damaging agents, and for increased cancer susceptibility to synthetic lethality. When Phase Ⅲ trials indicate which efforts at synthetic lethality are beneficial therapeutically, synthetically lethal down regulation of DNA repair pathways should be incorporated into standard medical treatments of cancers.

Evaluation of which DNA repair pathway(s) are epigenetically deficient in particular types of GI cancer and/or particular patients may prove useful in guiding choice of radiation, chemotherapeutic and/or synthetic lethality agent.

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