DNA alkylation and DNA methylation: cooperating mechanisms driving the formation of colorectal adenomas and adenocarcinomas?

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Defining our understanding of the association between DNA alkylation and colon carcinogenesis

Colorectal cancer has been called a disease of the somatic genome based on the fact that numerous somatic mutations have been identified in colorectal cancer, and these mutations have been shown to play a functional role in driving the formation of these cancers.^{1,2} More recently, a wealth of studies have implicated alterations in the epigenome, particularly aberrant CpG island DNA methylation, as also being important in cancer formation.³⁻⁵ Hence colorectal cancer is a disease that directly results from the serial accumulation of genetic alterations (for example, mutations in genes such as *APC*, *KRAS* and *TP53*) and epigenetic alterations (for example, aberrant methylation of *MLH1* and *CDKN2A*, etc.) in an evolving clone of colon epithelial cells, which in aggregate leads to the initiation and progression of neoplasms along a polyp to cancer progression sequence.⁶

In order to appreciate the significance of the epigenetic alterations in colorectal cancer, it is helpful to understand the nature of epigenetics. Epigenetics refers to heritable modifications to DNA that regulate gene expression. These heritable modifications are essentially amendments or chemical modifications to the DNA that include methylation of cytosine in CpG dinucleotides that are located in CpG islands, which are regions of DNA that have a high content of cytosines and guanines. The epigenetic status of the gene determines whether or not the gene can be expressed. Epigenetic regulation of genes normally plays a role in controlling cell differentiation, X chromosome inactivation and imprinting but is aberrant in cancer.

With regard to the factors that induce the genetic and epigenetic alterations observed in colorectal cancers, both endogenous cellular processes and environmental exposures play a role. The environmental factors that induce the

formation of cancer have been the subject of intense study, not only because their identification is expected to yield insights into the pathogenesis of colorectal cancer but also because they are modifiable factors that could be used in cancer prevention. Although a number of environmental risk factors (for example, smoking, cooked meat intake, body mass index) and preventive factors (for example, use of non-steroidal anti-inflammatory drugs, vegetable intake, physical activity) have been identified, the exact mechanisms by which they influence the development of colorectal cancer remain largely unknown. This may be a result of heterogeneity in colorectal carcinogenesis, resulting effectively in distinct phenotypes that are the result of different exposures.⁷⁻⁹ At the same time, inconsistencies may be attributed to the sheer number and complexity of potential environmental carcinogens to which people are exposed and to the difficulties in accurately determining the history of exposure in any individual.¹⁰ Nevertheless, strong evidence supporting the causative role of some of these suspected environmental agents has been derived from animal models which have been critical experimental systems for providing direct evidence for the role of specific mutagenic agents in cancer and for demonstrating dose-responses for suspected carcinogens with regard to their ability to cause DNA damage or cancer.¹⁰

Of the environmental factors, DNA alkylating agents are among the most consistently identified class of factors that cause DNA damage and cancer causing mutations in the colon. DNA alkylation refers to the addition of alkyl groups to specific bases, resulting in alkylation products such as O²-alkylthymine, O⁴-alkylthymine, O⁶-methylguanine and O⁶-ethylguanine, which cause DNA mutations.¹¹ Human colorectal DNA contains

O⁶-methylguanine (O6-MeG) and N⁷-methylguanine (N7-MeG), reflecting exposure to methylating agents, presumably found in foodstuffs, occupational chemicals, etc, as well as from endogenous alkylating agents and from in situ formation via nitrosation of amines.¹⁰ Whereas N7-MeG largely serves as a more robust biomarker of methylating exposures, O6-MeG is mutagenic and has been consistently associated with GC→AT transition mutations in genes such as *KRAS*.¹⁰ The association between oncogenic mutations in genes such as *KRAS* and DNA alkylation has provided strong evidence with regard to plausible molecular mechanisms through which this form of DNA damage could promote colorectal carcinogenesis in humans and for the potential role of DNA repair mechanisms that process alkylated DNA for serving as a protective barrier. Finally, consistent with the concept that DNA alkylation is a factor involved in cancer causation, increased O6-MeG levels have been identified in the distal colorectum, which is a common site for colon cancer, in the normal colorectum of people with colon cancer and in colorectal neoplasms that have *KRAS* mutations.¹²

One of the key enzymes involved in repairing O6-MeG is O6-methylguanine DNA methyltransferase (MGMT).¹¹ MGMT repairs the O6-MeG base through a stoichiometric process in which the methyl group on O6-MeG is transferred to a cysteine acceptor group which results in inactivation of the protein and subsequent ubiquitin mediated degradation.¹³ Thus continued expression of MGMT is required to maintain this DNA repair mechanism. As with O6-MeG levels, significant interindividual variability in MGMT activity of the colon has been observed. Notably, decreased MGMT activity has been shown in the normal mucosa of people with concurrent colorectal cancer.¹⁴ The study of Lees *et al*¹⁵ in this issue of *Gut* is the latest example of elegant work that has been conducted by these investigators¹⁰ that has defined our understanding of the association between DNA alkylation and colon carcinogenesis (see page 380). As mentioned above, Povey *et al* have demonstrated an association between MGMT activity and colon cancer in humans and in the 1,2 dimethylhydrazine mouse model of colon cancer. They have shown that low MGMT activity correlates in a linear fashion with increased cancer risk.¹⁰ Somewhat surprisingly, in light of this group's prior work and our understanding of the role of DNA alkylation in colon cancer, in the current study of individuals with colon adenomas compared with individuals with a normal colon, they have not

observed this inverse association, but instead have found increased MGMT activity in the normal mucosa of individuals with an adenoma compared with the normal mucosa of individuals without adenomas. Furthermore, they observed no difference in N7-MeG levels between cases and controls, and sub-stratification of cases by adenoma size or presence of GC-AT mutations in *KRAS* did not demonstrate any association. It is notable that N7-MeG was detected in all individuals, demonstrating exposure to alkylating processes regardless of adenoma status. In light of studies showing a correlation between low MGMT activity, increased O6-MeG concentrations and cancer of the bladder and lung, it is not clear why the results of Lees *et al* are not consistent with these from the same group of investigators that have shown that established colorectal adenocarcinomas associate with increased O6-MeG levels and decreased MGMT activity.¹⁴

Hence a major question raised by this study in relation to prior work is why individuals with colorectal adenomas have increased MGMT activity in the colorectal mucosa adjacent to the colorectal adenomas compared with age matched controls. The authors appropriately propose the possibility of polymorphisms in the promoter of *MGMT*

that may underlie the inconsistent results of studies of MGMT activity in people with adenomas compared with those with cancer. However, the low frequency of known *MGMT* polymorphisms in Western populations makes this an unlikely explanation.^{16–18} It is possible that functional polymorphisms in other gene that encode for proteins that interact with MGMT, such as *MSH2* or *MSH6*, may explain this discrepancy although this remains to be demonstrated.¹⁹ Other DNA repair enzymes that may play a role in confounding the relationship between MGMT activity, N7-MeG levels and colorectal neoplasms include *GSTT1*, *GSTM1*, *CYP2D6* and *CYP2E1*.²⁰ Povey *et al* have shown that homozygous *GSTT1*2* carriers are more likely than *GSTT1* carriers to have alkylated DNA and O⁶-methyldeoxyguanine (O6-MeG) in their colons and that *CYP2D6* poor metabolisers are more likely to have elevated MGMT activity and fewer tumours with *KRAS* mutations than *CYP2D6* extensive metabolisers or heterozygotes.²⁰ It is also possible that these differences in MGMT activity result in differing durations and consistency of exposure to DNA alkylating agents in individuals with colorectal adenomas compared with those with colorectal adenocarcinomas as increased exposure to DNA alkylating agents can increase

MGMT activity levels.²¹ Finally, the authors measured N7-MeG, rather than the primary mutagenic base, O6-MeG. Differences in the repair of these lesions may explain the lack of association between N7-MeG levels and *KRAS* mutations. The complexity and redundancy of the mechanisms that regulate the repair of DNA alkylation damage likely reflects the importance of this activity in maintaining the fidelity of the genome but is also a plausible source of the inconsistent associations between MGMT activity, levels of alkylated DNA and colorectal neoplasms.

Another interesting possibility that may explain these discrepant results is the effect of somatic events in the colorectal epithelium on DNA repair activity in the evolving tumours. Epigenetic alterations of *MGMT*, which result in its transcriptional silencing, have been identified in a variety of tumours.²² Thus it is possible that the association between MGMT activity and cancer risk may be more fully understood through a lens that includes somatic epigenetic alterations. *MGMT* is commonly aberrantly methylated in colorectal cancer and has been shown to be methylated in the earliest precursor of colorectal cancer, the aberrant crypt focus.^{23–24} Consistent with the possibility that somatic inactivation of MGMT plays a role in colorectal cancer predisposition, Shen *et al* detected aberrantly methylated *MGMT* in the normal colon mucosa from individuals with colon adenocarcinoma. They found that 50% of normal colons had detectable methylation of *MGMT* in patients with methylated *MGMT* in colorectal cancers compared with 12% of individuals with cancers that carried unmethylated *MGMT* and 6% of normal control colons.²⁵ These authors also found an association between *KRAS* GC→AT mutations and *MGMT* promoter methylation status in tumours. This observation raises the interesting possibility that a more direct association between DNA alkylation and cancer risk would have been apparent if DNA aberrantly methylated on CpG islands had been measured by the authors (see fig 1)

In order to understand the association between aberrant methylation of *MGMT* and DNA methylation caused by DNA alkylating agents, it is important to clarify that the aberrant methylation of *MGMT* refers to the formation of methylcytosine in CpG rich sequences of DNA, called CpG islands, as opposed to O⁶-methylation of guanine, which is the consequence of DNA alkylating agents and the subject of the studies by Lees *et al*.¹⁵ CpG DNA methylation is an epigenetic mechanism for regulating gene expression that is driven by the DNA methyltransferases,

Figure 1 Model of relationship between DNA repair mechanisms, DNA alkylation and colorectal cancer. Aberrant methylation of CpG islands located in the promoter region of DNA repair genes such as *MGMT* or *MLH1* occurs in the colon epithelial cells resulting in gene silencing of these genes and decreased ability of the colon epithelial cells to repair DNA damage. The alkylated base, O6-methylguanine, is mutated to adenine, which can induce oncogenic mutations in genes such as *KRAS*. These mutations increase future cancer risk. In addition to tissue based silencing of DNA repair genes, functional polymorphisms in these genes are thought to be responsible for differences between individuals' ability to repair DNA alkylation damage in the colon. It is possible that these different aetiologies for mutations play a role in the molecular subgroups of colon cancer that are currently recognised, including microsatellite unstable cancer, chromosomal unstable cancer and CpG Island methylator phenotype cancer.

DNMT1, DNMT3a and DNMT3b, and is a normal process involved in the regulation of genes in differentiated tissue and during development, which becomes aberrantly regulated in cancer. CpG island DNA methylation regulates gene expression, presumably in conjunction with histone modifications. In contrast, as explained above, O⁶-methylguanine and N⁷-methylguanine are the direct result of DNA alkylating and nitrosating agents in the environment, such as tobacco, and one of the products of these DNA alkylating events is the mutagenic base O⁶-MeG. The argument for aberrant methylation of *MGMT* as a mechanism for impairing the repair activity of DNA alkylation in colon tissue and creating a predisposition to colorectal neoplasm formation is supported not only by the studies of Shen *et al* but also by the observation of aberrantly methylated genes, including *MGMT*, *MLH1*, *ESR1* and *MYOD*, in the normal colon mucosa of elderly individuals and in the histologically normal colon mucosa individuals with ulcerative colitis (a condition that increases the risk of colon cancer).^{25–27} These observations suggest that aberrant DNA methylation can be one of the earliest events observed in cancer, raising the possibility that one method by which epigenetic alterations may promote cancer formation is through inactivating DNA repair genes, such as *MGMT*. In fact, one of the particularly provocative aspects of recent studies of epigenetic alterations in cancer is the observation that a number of genes that are involved in DNA repair are commonly found to be aberrantly methylated in the early stages of tumours.²³ Aberrant DNA CpG island methylation of DNA repair genes, such as *MGMT*, may predispose clones of cells in at-risk tissues to accumulate DNA damage, such as DNA alkylation, with an increased risk of mutagenicity. In a similar vein, the aberrant methylation of *MLH1*, a member of the DNA mismatch repair machinery, has been found in approximately 10% of colon cancers,²⁸ which results in the mismatch repair deficiency phenotype, microsatellite instability, in the absence of any germline mutation in the mismatch repair genes.

Finally, Lees *et al* suggest that their findings may indicate differences in the role of *MGMT* depending on the stage of colorectal carcinogenesis.¹⁵ This explanation is also biologically plausible as the effects of different oncogenes and tumour suppressor genes on the behaviour of cancer cells have been shown to differ depending on whether the cancers are

early or advanced.²⁹ It appears that the conflicting results observed in human studies may only be resolved using an integrated analysis that takes into account the functional polymorphisms in the cooperating enzymes involved in the repair of DNA alkylation, the degree and timing of exposure to DNA alkylating agents and the somatic inactivation of DNA repair mechanisms through epigenetic mechanisms in at-risk tissues.

Gut 2007;**56**:318–320.
doi: 10.1136/gut.2006.106849

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Sources of support/acknowledgements: This work was supported by a Lilly-Damon Runyon Clinical Investigator Award from the Damon Runyon Cancer Research Fund, a VA Presidential Early Career Award for Scientists and Engineers from the Department of Veterans Affairs R&D Service, from a pilot project award from Early Detection and Intervention Initiative (FHCRC Comprehensive Cancer Center Support Grant) (to WMG), and R01 CA 114467 and R01 CA 59045 (to CMU and John Potter).

Competing interests: None.

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