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Hunterian Lecture

Epigenetics, mismatch repair genes and colorectal cancer

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The recent discovery of hypermethylation of the promoter of genes is a powerful epigenetic mechanism for the inactivation of tumour suppressor genes in colorectal and other cancers. Approximately 95% of hereditary non-polyposis colorectal cancers (HNPCCs) and 15% of sporadic colorectal cancers (CRCs) are replication error positive (RER$^+$). Although DNA mutations are found in mismatch repair genes in the majority of HNPCC CRC, mutations are rare in sporadic RER$^+$ CRCs. We have shown that the principal cause of an RER$^+$ phenotype is hypermethylation of the promoter of hMLH1, resulting in the absence of hMLH1 protein. In contrast to sporadic RER$^+$ CRCs, we found that hypermethylation of hMLH1 does not occur in HNPCC CRC, suggesting the possibility of further differences between the two types of RER$^+$ tumours in the adenoma to carcinoma pathway. Other known tumour suppressor genes with few or no mutations may be candidates for epigenetic changes. One such gene is E-cadherin, and we described the first mutations of this gene in CRCs. Half of all CRCs were found to be hypermethylated in the E-cadherin promoter and this correlated with reduced E-cadherin expression. Epigenetic changes occur in CRCs and arise in different frequencies in separate genes. Hypermethylation of the promoter may be reversed and gene function restored to a cell, thus partially undoing the cancer phenotype.

Key words: Epigenetics – Mismatch repair genes – Colorectal cancer – Hypermethylation – Replication error

Early in the last century, Warthin described several families who appeared to have a predisposition to cancer.[1] In 1966, Lynch and Shaw published their findings on two large families with a number of individuals having multiple primary cancers. This work prompted interest from French, who succeeded Warthin. He gave custody of all the records and pathology specimens which had been collected by Warthin to Lynch who produced an updated review of cancer family 'G' and demonstrated an autosomal-dominant pattern of inheritance with the majority of cancers being adenocarcinomas of the colon, endometrium and stomach.

By the 1980s, many reports of a 'cancer family syndrome' were appearing in the medical literature. This then became subdivided into Lynch syndrome I (families with mainly CRCs at an early age) and Lynch syndrome II (families with CRCs and extracolonic cancers). This was eventually clarified with the introduction of the term hereditary non-polyposis colorectal cancer (HNPCC) to emphasise the lack of multiple colonic polyps and to separate it from the polyposis syndromes. The 'Amsterdam criteria' for families with hereditary non-polyposis colorectal cancer were established by the International Collaborative Group on HNPCC (ICG-HNPCC).[2,3]

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The HNPCC susceptibility loci were mapped to chromosome 2p16 and chromosome 3p21 following the study of large kindreds.[1] Expanded microsatellites were found in HNPCCs where regions of loss had been expected, and this was termed microsatellite instability.[4] This was followed by identification of the hMSH2 gene on chromosome 2p,[5,6] and the hMLH1 gene on chromosome 3p.[7,8] Mutations in hMSH2 and hMLH1 account for the majority of reported HNPCC cases. These genes, and the proteins they encode for, are responsible for eukaryotic mismatch repair.

Microsatellite instability

Microsatellites are repetitive genetic loci which are normally relatively stable, and microsatellite instability (or replication error positive, RER^+) is defined as a relatively frequent change of any length of these loci, due to either insertion or deletion of repeated units. They are prone to slippage during DNA replication and this results in a small loop in either the template or nascent DNA strand. These are normally repaired; however, in the absence of efficient mismatch repair function, these 'loops' may become permanent, and alleles of different sizes will be formed at the next round of replication.

Microsatellite instability is seen when new alleles are formed relatively frequently at microsatellite loci in tumour DNA when compared with the two parental alleles in the normal DNA of the patient. Multiple, different sized alleles may accumulate over several generations if a cancer has a mutated mismatch repair gene. While microsatellite instability is seen in about 90% of all HNPCC CRC and at the majority of microsatellite loci,[4] it is only seen in 10–15% of sporadic CRCs.

RER^+ cancers tend to be diploid or near diploid karyotype with similar mutations in the APC and K-Ras genes but reduced mutations in p53. Mutations found in RER^+ cancers but not RER^- cancers include the TGF-β, IGFII, BAX and E2F4 genes.

Sporadic colorectal cancer and microsatellite instability

It has been previously estimated that HNPCC is responsible for approximately 5% of all CRCs. However, a prospective study from Finland has demonstrated that only 10 of 509 (2%) consecutive CRC patients had germline mutations in either hMLH1 or hMSH2.[9]

As per Knudson's hypothesis for tumour suppressor genes,[10] microsatellite instability is the result of inactivation of both alleles of a mismatch repair gene. In HNPCC CRC, a single mutation is inherited in the germline, and microsatellite instability only follows inactivation of the other allele. In sporadic RER^+ cancers, inactivation of both alleles must occur somatically before microsatellite instability is observed.[11]

Although germline mutations in hMLH1 and hMSH2 are commonly found in HNPCC CRC, somatic mutations have not been described frequently in sporadic RER^+ CRC.[1] This led to the hypothesis that either other genetic loci or epigenetic mechanisms were responsible for microsatellite instability.[11] However, Thibodeau *et al*.[12] were able to demonstrate that 40 of 42 (95%) of sporadic RER^+ CRCs lacked expression of either hMLH1 or hMSH2, and that hMLH1 was the altered protein in 95% of these cases. They concluded that hMLH1 has a principal role in the phenotype of sporadic RER^+ CRCs.

Epigenetics

While genetics is the scientific study of heredity, epigenetics is the study of the mechanisms by which genes bring about their phenotypic effects. There is currently a tremendous interest in the field of epigenetics and this is focused on the relationship between chromatin structure and DNA methylation patterns.

Inactivation of several tumour suppressor genes by an epigenetic process that involves hypermethylation of the promoter region has been described.[13–16] The first links between microsatellite instability and hypermethylation of promoter DNA followed the discovery that both endogenous and exogenous DNA sequences were more likely to be hypermethylated in sporadic RER^+ CRCs.[17] This finding together with so few somatic mutations being described in sporadic RER^+ CRC, led to the discovery of hypermethylation of the promoter region of hMLH1 being associated with lack of hMLH1 protein expression and microsatellite instability.[18]

Initially, we set out to investigate the complete mechanisms of inactivation of mismatch repair genes that may result in the RER^+ phenotype in sporadic CRCs.

Inactivation of mismatch repair genes in sporadic RER^+ CRCs

Ten of 42 CRCs (cell lines) studied (24%) were RER^+. We detected mutations in hMLH1 in five of the ten RER^+ cancers (50%), and determined that the hMLH1 promoter was hypermethylated in five of the ten RER^+ cancers (50%) and in none of the RER^- cancers ($P = 0.0004$).[19] Three of the 10 RER^+ cancers (30%) had both a mutation and hypermethylation of the promoter of hMLH1. Therefore, inactivating mutations or hypermethylation of the promoter of hMLH1 was found in 7 of the 10 RER^+ cancers (70%). Hypermethylation of the hMLH1 promoter was associated with absent protein expression.

Table 1 Inactivating mutations or hypermethylation of the promoter of hMLH1 was found in 7 of the 10 RER⁺ cancers (70%)

Cell line	Mutation hMLH1	hMSH2	Methylation hMLH1
VACO5	Yes		Yes
SW48			Yes
LS411	Yes		Yes
LS174T			
LOVO		(Yes)*	
HCT116	Yes		
HCA7			Yes
DLD1/HCT15*			
GP2d/5d	Yes		
LS180	Yes		Yes

*Together with the knowledge that the LOVO cell line has an exonic deletion in hMSH2 and the DLD1/HCT15 cell line is known to have a mutation in GTBP/MSH6, we were able to explain the RER⁺ phenotype in 9 of the 10 (90%) RER⁺ cancers.

Together with the knowledge that the LOVO cell line has an exonic deletion in hMSH2 and the DLD1/HCT15 cell line is known to have a mutation in GTBP/MSH6, we were able to explain the RER⁺ phenotype in 9 of the 10 (90%) RER⁺ cancers (Table 1). Using a demethylating agent, 5-azacytidine, we were able to demethylate the hMLH1 promoter region in hypermethylated RER⁺ cell lines with subsequent re-expression of the hMLH1 protein. Others have shown hypermethylation of the hMLH1 promoter in up to 84% of sporadic RER⁺ CRCs.[20]

Therefore, mutations of hMLH1 together with hypermethylation of the hMLH1 promoter region are the principal cause of the mutator phenotype in the majority of sporadic RER⁺ CRCs.

Is there hypermethylation of the hMLH1 promoter in HNPCC CRCs?

Although Knudson's hypothesis has traditionally focused on mutations in the DNA sequence and on loss of heterozygosity (LOH), it has been proposed the hypothesis should be expanded to include epigenetic mechanisms of gene inactivation, such as hypermethylation of the promoter.[13]

With the knowledge that germline mutations of hMLH1 and hMSH2 are responsible for the majority of CRCs in HNPCC patients and that sporadic RER⁺ CRCs are the result of hypermethylation of the hMLH1 promoter, we investigated the possible second hit mechanism of the hMLH1 gene in CRCs from HNPCC patients. DNA was available from the CRCs of 10 HNPCC patients and from 10 sporadic RER⁺ CRCs from Helsinki, Finland. All tumours were confirmed to be RER⁺. All 10 HNPCC patients had hMLH1 germline mutations. No mutations in hMLH1 or hMSH2 were detected in patients with sporadic RER⁺ CRCs. No hypermethylation was detected in the HNPCC CRC compared with 7 of the 10 sporadic RER⁺ CRCs (70%; $P < 0.002$).[21] LOH at the hMLH1 locus was found to be the second hit mechanism in 8 of the 10 HNPCC CRCs (80%; Table 2). Therefore, hypermethylation of the promoter of hMLH1 is not a common second hit mechanism in CRCs from HNPCC patients.

Table 2 Hypermethylation of the promoter of hMLH1 is not a common second hit mechanism in CRCs from HNPCC patients

	HNPCC	RER⁺	*P*
Median age (years)	58	79	< 0.05
hMLH1 mutation	10	0	
hMLH1 hypermethylation	0	7	< 0.002
LOH	8	ND	

ND, not determined.

CpG island methylator phenotype (CIMP)

A CpG island methylator phenotype (CIMP) has recently been proposed in CRCs and gastric cancers.[22] CRCs with a methylator phenotype were found to have a high frequency of hypermethylation of the promoter region of the p16 and THBS1 genes. This included most RER⁺ cancers with hMLH1 hypermethylation.

Recently, Yamamoto *et al*.[23] extended our work on the different mechanisms of hMLH1 inactivation between HNPCCs and sporadic RER⁺ CRCs by looking at the CIMP in the two groups. They looked at the methylation status of several target genes and found that while only 23% of HNPCC CRCs were CIMP⁺, 53% of sporadic RER⁺ CRCs were CIMP⁺ ($P = 0.018$).

Mismatch repair genes and the 'adenoma-carcinoma sequence'

It has been proposed that HNPCC cancers and sporadic RER⁺ CRCs develop along a different pathway to other CRCs, and these cancers are said to have an increased mutation rate or a 'mutator phenotype' as a result of inactivation of a mismatch repair gene.

Some HNPCC cancers may indeed have an accelerated pathway to carcinoma, when the loss of the remaining wild-type allele occurs early and before inactivation of the adenomatous polyposis coli (APC) gene. There is evidence to suggest that APC mutations remain the initiating event in sporadic RER⁺ colon cancers,[24] although there may be an increased mutation rate in HNPCC cancers in which the mismatch repair gene is inactivated prior to APC mutations (Fig. 1).

Hypermethylation of the E-cadherin promoter in CRCs

E-cadherin is a recognised tumour suppressor gene and an ideal candidate gene to investigate for genetic

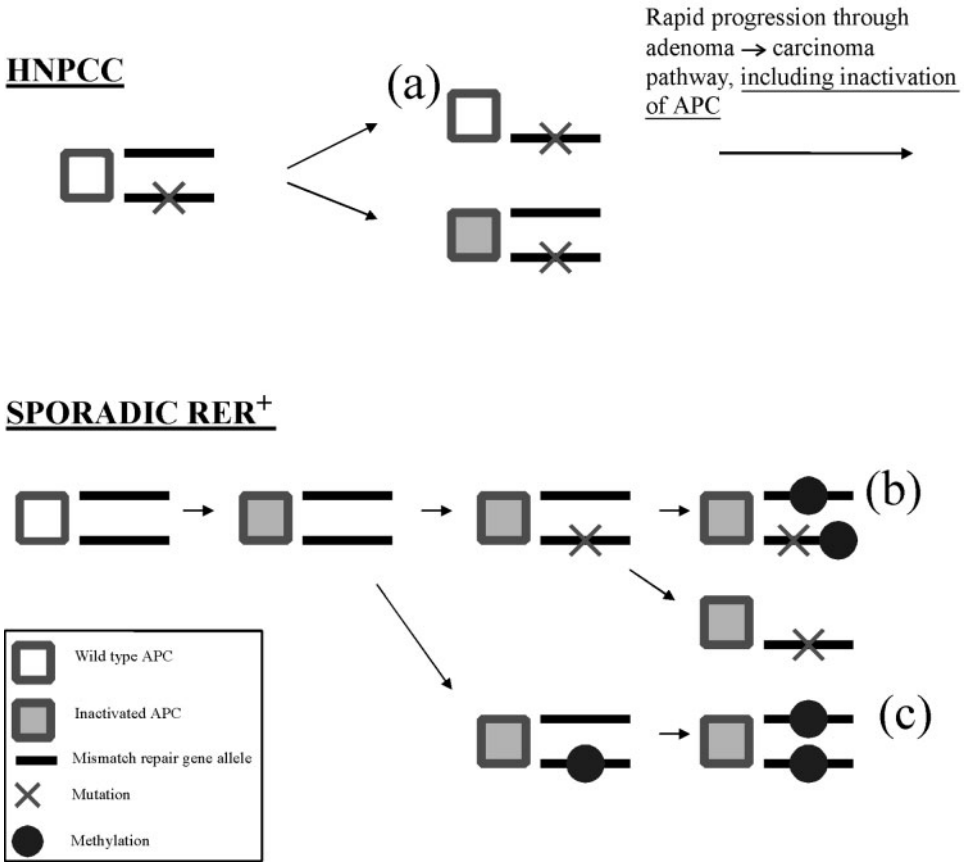

Figure 1 Model for adenoma to carcinoma pathway in HNPCCs versus sporadic RER+ colorectal cancers. In HNPCCs, a germline mutation is present in every cell, and only one further event (usually LOH) is required to inactivate a mismatch repair gene. This may occur at an early stage (a), before inactivation of APC, and result in a rapid progression through the adenoma to carcinoma pathway (the mutator phenotype). In contrast, inactivation of a mismatch repair gene in sporadic RER+ cancers is likely to be a late event, after inactivation of APC. Although, inactivation may be due to a somatic mutation (with LOH), hypermethylation of the hMLH1 promoter region is the commonest cause of inactivation of mismatch repair genes in these cancers, and is usually a bi-allelic event (b,c). [*Gut* 2000; **47**: 148–153, Figure 3 reproduced with permission from the BMJ Publishing Group.]

mutations and hypermethylation changes, as although its expression is known to be decreased in invasive CRC, no mutations had previously been described in CRCs.

E-cadherin protein is the prime mediator of epithelial cell-to-cell adhesion through the interactions of its extracellular domain. The APC protein competes directly with E-cadherin for binding to β-catenin and, therefore, may be an indirect regulator of E-cadherin mediated adhesion.

A total of 42 CRCs (cell lines) were studied. We detected 4 mutations in 3 of the 10 RER+ cancers (30%) and none of the RER− cancers (7% overall). LS174T was found to have 2 mutations with no subsequent protein expression.[25] It is also noteworthy that LS174T has no mutation in either APC or β-catenin, and the E-cadherin mutation may be an alternative early change in the adenoma to carcinoma pathway.

Hypermethylation of the E-cadherin promoter region has been described in breast, prostate, hepatocellular and thyroid cancer. As we had only found a low incidence of E-cadherin mutations in CRCs, we then hypothesised that hypermethylation of the E-cadherin promoter may be the cause of decreased E-cadherin protein expression in CRC. A panel of 28 CRCs were studied. Although three of the 28 cancers were RER+ (11%), no mutations were detected.

Table 3 Thirteen of the 28 CRCs (46%) were found to have hypermethylation of the E-cadherin promoter region. This was significantly associated with reduced immunoexpression of the E-cadherin protein (grade 0/1), P = *0.036*

	Grade 0/1 (0–25%)	Grade 2/3 (> 25%)
Hypermethylated E-cadherin	8	5
Unmethylated E-cadherin	3	11

However, 13 of the 28 CRCs (46%) were found to have hypermethylation of the E-cadherin promoter region.[26] This was significantly associated with reduced immunoexpression of the E-cadherin protein ($P = 0.036$; Table 3).

More recently, Azarscab *et al*.[27] have studied hypermethylation of the E-cadherin gene in biopsy samples taken at colonoscopy in patients with ulcerative colitis. They found evidence of E-cadherin hypermethylation in 93% of patients with dysplasia compared with 6% of patients without dysplasia and 0% of control patients. They have suggested that hypermethylation of the E-cadherin gene may be an attractive new marker of detecting ulcerative colitis patients with a high risk of CRCs.

Adenocarcinoma of the small intestine

More recently, hypermethylation of the hMLH1 promoter has been described in up to 77% of sporadic RER$^+$ endometrial carcinomas[28] and up to 100% of sporadic RER$^+$ gastric carcinomas[29] together with reduced expression of the hMLH1 protein.

We hypothesised that hypermethylation of the hMLH1 gene would be responsible for microsatellite instability in sporadic RER$^+$ small intestine adenocarcinomas. The RER status of 21 non-familial, non-ampullary adenocarcinomas was determined. Unfortunately, only one of the cancers (5%) proved to be RER$^+$, and this cancer was found to express both hMLH1 and hMSH2 protein on immunohistochemistry. However, as this was a large collection of an unusual cancer, we screened the tumours for mutations in the mutation cluster region (MCR) of the APC gene. The MCR is responsible for 60% of mutations in sporadic CRCs.

No mutations were detected in the MCR of the APC gene.[30] On immunohistochemical staining, β-catenin showed increased nuclear expression with loss of membranous staining in 10 cancers (48%), and absent or decreased membrane expression of E-cadherin was found in 8 cancers (38%). Together, this may reflect an early alternative to APC mutations in the genetic pathway of adenocarcinoma of the small intestine. It is possible that APC inactivation may still occur, but secondary to epigenetic changes such as hypermethylation of the APC promoter region.

Esteller *et al*.[31] have recently described APC hypermethylation in a number of cancers including CRCs, and gastric, pancreatic, liver and oesophageal cancer. They reported hypermethylation of the APC gene in 18% of CRCs and adenomas suggesting that the epigenetic change occurs early in the genetic pathway. This change was associated with reduced protein expression and did not occur in the tumours of FAP patients with germline mutations. This is analogous to our finding of no hypermethylation of hMLH1 in the CRC of HNPCC patients.

The future of epigenetics

There are potentially a large number of unrecognised tumour suppressor genes awaiting discovery. This will be facilitated by new genomic scanning techniques which can rapidly detect aberrant methylation patterns. It is possible that known tumour suppressor genes in which no mutations are reported in certain cancers will be found instead to be hypermethylated.

In vitro studies have shown how hypermethylated cancers may potentially be demethylated with re-expression of the relevant protein. This has led to work on the therapeutic reversal of DNA hypermethylation in cancers with deficient mismatch repair as they are associated with anticancer drug resistance.[32]

Conclusions

Mutations of hMLH1 together with hypermethylation of its promoter region are the underlying cause of the mutator phenotype in the majority of sporadic RER$^+$ CRCs. However, hypermethylation of the hMLH1 promoter is not the 'second hit' mechanism of inactivation of mismatch repair genes in HNPCC CRCs.

The first known mutations of the E-cadherin gene in human CRCs were described. Hypermethylation of the promoter region of the E-cadherin gene in CRC was discovered and was shown to correlate significantly with reduced E-cadherin protein expression.

The low frequency of the RER$^+$ phenotype prevented further investigation of the role of mismatch repair genes in adenocarcinomas of the small intestine. It is significant that no mutations were detected in the mutation cluster region of the APC gene, and it would be interesting to investigate the hypermethylation status of the APC gene in these and other cancers.

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