



**HAL**  
open science

## DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch Syndrome

George Poulgiannis, Ian Frayling, Mark Arends

► **To cite this version:**

George Poulgiannis, Ian Frayling, Mark Arends. DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch Syndrome. *Histopathology*, 2009, 56 (2), pp.167. 10.1111/j.1365-2559.2009.03392.x . hal-00549900

**HAL Id: hal-00549900**

**<https://hal.science/hal-00549900>**

Submitted on 23 Dec 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



**DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch Syndrome**

Journal:	<i>Histopathology</i>
Manuscript ID:	HISTOP-06-09-0373
Manuscript Type:	Review
Date Submitted by the Author:	30-Jun-2009
Complete List of Authors:	Poulogiannis, George; Harvard Medical School, Department of Systems Biology and Division of Signal Transduction, Beth Israel Deaconess Medical Center; University of Cambridge, Dept of Pathology Frayling, Ian; University Hospital of Wales, Institute of Medical Genetics Arends, Mark; University of Cambridge, Department of Pathology
Keywords:	Mismatch repair deficiency, Microsatellite instability, Lynch Syndrome, Colorectal cancer



Formatted: Line spacing: Double

REVIEW

## DNA mismatch repair deficiency in sporadic colorectal cancer and Lynch syndrome

George Pouligiannis,<sup>1,2</sup> Ian M Frayling<sup>3</sup> & Mark J Arends<sup>1</sup>

Deleted: ,

Deleted: ,

<sup>1</sup>Department of Pathology, University of Cambridge, Cambridge, UK,

Deleted: Affiliations:¶

<sup>2</sup>Department of Systems Biology, Harvard Medical School and Division of SignalTransduction, Beth Israel Deaconess Medical Center, Boston, MA, USA [and](#)<sup>3</sup>Institute of Medical Genetics, University Hospital of Wales, Cardiff, UK[Address for correspondence;](#)

Deleted: Correspondence

Deleted: to

Formatted: Font: Not Bold

Formatted: Font: Not Bold

Deleted: Tel: 01223 217813, Fax: 01223 586770, Email

Dr M. J. Arends, Department of Pathology, University of Cambridge, Box 235,

Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2QQ, UK. [e-mail:](#)

mja40@cam.ac.uk

**Running title:** *Mismatch repair deficiency in colorectal cancer*

Formatted: Font: Italic

**Keywords:** [colorectal cancer](#), [Lynch syndrome](#), [microsatellite instability](#), mismatch

Formatted: Font: Not Bold

repair deficiency.

Deleted: , Microsatellite instability, Lynch Syndrome, Colorectal cancer

Abbreviations: 5FU, 5-fluorouracil; B2M,  $\beta_2$ -microglobulin; CIN, chromosomal instability; CRC, colorectal cancer; HNPCC, hereditary non-polyposis colorectal cancer; IDL; insertion/deletion loop; LS, Lynch syndrome; MMR, mismatch repair; MSI, microsatellite instability; MSI-H, high-frequency microsatellite instability; MSI-L, low-frequency microsatellite instability; MSS, microsatellite stable; NER, nucleotide excision repair; XP, xeroderma pigmentosum

DNA mismatch repair (MMR) deficiency is one of the best understood forms of genetic instability in colorectal cancer (CRC), and is characterized by the loss of function of the MMR pathway. Failure to repair replication-associated errors due to a defective MMR system allows persistence of mismatch mutations all over the genome, but especially in regions of repetitive DNA known as microsatellites, giving rise to the phenomenon of microsatellite instability (MSI). A high frequency of instability at microsatellites (MSI-H) is the hallmark of the most common form of hereditary susceptibility to CRC, known as Lynch syndrome (LS) (previously known as hereditary non-polyposis colorectal cancer syndrome), but is also observed in ~15-20% of sporadic colonic cancers (and rarely in rectal cancers). Tumour analysis by both MMR protein immunohistochemistry and DNA testing for MSI is necessary to provide a comprehensive picture of molecular abnormality, for use in conjunction with family history data and other clinicopathological features, in order to distinguish LS from sporadic MMR-deficient CRC. Identification of the gene targets that become

Deleted: Abstract¶

Deleted: characterised

Deleted: (HNPCC)

Deleted: -

Deleted: -

Deleted: Lynch Syndrome

mutated in MMR-deficient tumours may explain, at least in part, some of the clinical, pathological and biological features of MSI-H CRCs and holds promise for developing novel therapeutics.

### Introduction

Both epidemiological<sup>1</sup> and *in vitro*<sup>2</sup> studies have shown that oncogenic transformation requires the accumulation of at least 6–12 mutational events, possibly more,<sup>3, 4</sup> a process that is facilitated by some form of genetic instability<sup>5, 6</sup>. Recent advances in high-throughput sequencing<sup>7–9</sup> have also shown that mutations in DNA repair and replication genes are present in >58% of cancer cell lines, suggesting that the genetic instability derived from these changes is an intrinsic feature of most cancers. It is by no means clear whether genetic instability in itself is a phenotype that is selectable in tumour cells, or whether it is merely an epiphenomenon—a marker of some other selectable advantage conferred by loss of a DNA repair function. Interestingly, base-to-base mismatches (e.g. A-C instead of A-T) that are not repaired because of deficiency of the DNA mismatch repair (MMR) system, which are also known as mismatch repair abnormalities, usually occur at later rather than early stages of tumour progression,<sup>4</sup> suggesting that normal mutation rates are not necessarily growth-limiting during the early stages of the tumorigenic process. *In silico*

Formatted: Font: Not Italic

Deleted:

Deleted:

Formatted: Font: Italic

Deleted: -

Deleted: more

Deleted: -

Deleted: u

modelling has suggested that mutation rate *per se* is of much less value to a developing tumour than growth advantage<sup>10</sup>. Loss of the MMR system interferes with the induction of apoptosis by some forms of DNA damage and thus enhances cell survival after such DNA damage that is usually recognized by the MMR system<sup>11,12</sup>. Hence, MMR deficiency may primarily confer a selectable growth advantage in the form of reduced susceptibility to apoptosis, with an increased mutation rate as a secondary and perhaps only weakly selectable phenotype<sup>11,13</sup>.

Deleted: recognised

Deleted: -

Most conditions associated with loss of a DNA repair function are manifest by recessive inheritance of two inactivated alleles for the phenotype to be evident, but the phenotype is not manifested in carriers, such as xeroderma pigmentosum (XP). Moreover, mutations of XP genes are not observed in sporadic forms of the tumours observed in XP patients<sup>14</sup>. In distinct contrast, Lynch syndrome (LS)—a syndrome of inherited susceptibility to adenocarcinomas of the colorectum and endometrium along with tumours at a range of other sites (adenocarcinomas of ovary, stomach and small intestine; transitional cell carcinomas of ureter and renal pelvis; sebaceous tumours of skin; and gliomas of brain) due to defective DNA MMR in tumour cells—is characterized by dominant inheritance, with all somatic cells possessing one mutated and one wild-type MMR allele, and thus at the level of the individual cell, there appears to be a selectable advantage to a developing tumour cell in having inactivation of the second of its two MMR alleles, such as the diminution of susceptibility to apoptosis. The recognition of a severe condition due to inheritance of two mutated MMR alleles (sometimes manifest as Turcot's syndrome), which is similar in character to other recessive DNA repair deficiency syndromes, and distinct from classical LS, is further evidence that LS is an unusual exception in the range of DNA repair deficiency syndromes<sup>15</sup>.

Deleted: Syndrome

Deleted: -

Deleted: mismatch repair

Deleted: -

Deleted: characterised

Deleted: .

Deleted: Syndrome

Deleted: Lynch Syndrome

Deleted: Lynch Syndrome

Loss of DNA MMR function can be caused by either mutations or deletions in, or epigenetic silencing of, both copies of one of the MMR genes, the major ones being MSH2, MLH1, MSH6 and PMS2. The resulting failure to repair replication-associated errors produces a high frequency of mutations, either single base mismatches or in regions of short tandem DNA repeats (the repeat units often being 1–4 bp in length), known as microsatellites <sup>16</sup>.

Deleted: mismatch repair

Deleted: either

Deleted: :

Formatted: Font: Not Italic

Formatted: Font: Not Italic

Deleted: or

Deleted: -

High-frequency microsatellite instability (MSI-H) is the hallmark of cancers in LS [previously known as hereditary non-polyposis colorectal cancer (HNPCC) syndrome,<sup>17</sup>] and is also observed in ~15–20% of sporadic colonic cancers. Some examples of microsatellite instability (MSI) at individual mono- and dinucleotide loci are shown in Figure 1. Although loss of MMR, and thus the development of MSI, is observed in rectal cancers, this is a rare phenomenon associated almost entirely with LS: the majority of colorectal cancers (CRCs) exhibiting MSI develop in the colon, particularly in the right side of the large bowel (caecum, ascending colon and transverse colon) <sup>18</sup>. The underlying hypothesis is that this MMR deficiency mediates an increase in the mutation rate that produces a “mutator phenotype”, which leads to mutations accumulating in genes that play regulatory roles in the growth of normal colonic epithelia, and these are selected during carcinogenesis as part of a progressive release from normal growth restraints.

Deleted: Lynch Syndrome

Deleted: (

Deleted:

Deleted: )

Deleted: -

Deleted: Figure

Deleted: Lynch Syndrome

Deleted: -

### *The molecular basis of MMR deficiency*

CRC is associated with two forms of genetic instability: chromosomal instability (CIN) and microsatellite instability (MIN or MSI) <sup>19</sup>. In contrast to the molecular basis underlying CIN, which is not precisely defined, although it may well involve

Deleted: Colorectal cancer (

Deleted: )

Deleted: ,

mutant *APC*, as *APC* is involved in spindle formation and correct chromosomal segregation, MSI is almost always associated with defective function of a small number of proteins, the products of the MMR genes *MLH1*, *MSH2*, *MSH3*, *MSH6*, *PMS1* and *PMS2*<sup>20,21</sup>.

Deleted: mismatch repair

Based on their MSI status, tumours can be divided into three categories: (i) those that show no MSI, termed microsatellite stable (MSS); (ii) tumours in which MSI is found at a high frequency (defined as  $\geq 29\%$ ) of microsatellites tested (MSI-H); and (iii) tumours showing MSI at a low frequency (MSI-L) of microsatellite markers tested

Deleted: a

Deleted: microsatellite instability

Deleted: b

Deleted: c

(1-28%, equivalent to one to two out of 10 markers). There has been an ongoing debate as to whether MSI-L tumours exist as a distinct entity. Two separate studies<sup>22,</sup>

Deleted: -

Deleted: 1 -

Deleted: 2

<sup>23</sup> showed that if enough microsatellite markers are tested, eventually one is found to be unstable, and hence all or almost all tumours exhibit at least MSI-L if enough microsatellites are analysed. Previous studies have also shown that MSI-L tumours appear phenotypically similar to the MSS tumours, so they are often categorized

Deleted: categorised

together with the MSS group. Thus, MSI-H is due to defective MMR and can be considered as either “Lynch syndrome-associated MSI” occurring due to inheritance of a MMR gene mutation, or “sporadic MSI” occurring almost always due to hypermethylation of the *MLH1* promoter (see below). However, there is evidence that a tumour phenotype manifesting MSI-L may be associated with sporadic colorectal tumorigenesis and a cancer predisposition condition distinct from LS<sup>24,25</sup>.

Deleted: Syndrome

Deleted: tumorigenesis

Deleted: Lynch Syndrome

The components of the MMR system are highly conserved in both pro- and eukaryotic organisms. Upon DNA damage as a result of oxidative stress, lipid peroxidation or other forms of endogenous stress, or following replication-associated errors, MMR proteins can recognize both base mismatches and insertion/deletion loops (IDLs, which are functionally equivalent as an insertion on one strand has the

Deleted: recognise

Deleted:



same effect as a deletion of the same number of nucleotides on the other strand) and act to repair these abnormalities. MMR also readily acts to repair some damaged bases, which are thus, by definition, mismatched. In eukaryotes (Figure 2), recognition of mismatches and single base IDLs depends upon heterodimeric complexes of MutS-related proteins: MSH2–MSH6 (known as hMutSAlpha). In contrast, IDLs of two to eight nucleotides are recognized by the complex MSH2–MSH3 (known as hMutSBeta). There is overlap in the specificities of these two complexes and hence some redundancy in their activity. A second type of heterodimeric complex, involving two MutL-related proteins MLH1 and PMS2 (hMutLAlpha) or MLH1 and PMS1 (hMutLBeta) binds to the MSH-containing complexes along with certain replication factors and other proteins, so that excision and resynthesis can proceed. As MSH2 and MLH1 are the common components of these complexes, then loss of either will abrogate all MMR activity, whereas loss of one of the other components, such as MSH6, will only diminish the activity of MMR against base mismatches or single nucleotide IDLs. This is probably why most LS families are found to have a germ-line mutation in either *MSH2* or *MLH1*, whereas mutant *MSH6* families are less common and have a somewhat different propensity to colorectal vs. endometrial cancer<sup>26</sup>. MMR components also interact functionally with some nucleotide excision repair (NER) proteins to contribute to the resolution of Holliday junctions<sup>27</sup>. The MSH2 and MSH3 proteins, through interaction with the NER complex Rad1–Rad10, are involved in the removal of duplicated DNA sequences greater than 30 bases in DNA double-strand break repair.

Deleted: Figure

Deleted: insertion/deletion loop

Deleted: -

Deleted: 2-8

Deleted: recognised

Deleted: -

Deleted: Lynch Syndrome

Deleted: ersu

Deleted: -

Deleted: ersu

*MMR defects in sporadic vs. hereditary CRCs*

CRC is traditionally divided into two main types: familial, those cases with either some degree of family history of, or obvious familial predisposition to the disease (~20–25%); and sporadic, corresponding to those cases without evidence of genetic or familial predisposition to the disease (~75–80%). MSI at high frequency caused by a defective MMR system is a hallmark of the most common hereditary form of CRC, previously known as HNPCC, but which has now reverted to being called Lynch syndrome<sup>17, 28</sup>. LS sufferers inherit one germ-line mutation in an MMR gene and develop tumours from clonal expansion of those cells acquiring a second hit, mutation or loss or epigenetic inactivation, of the remaining wild-type allele resulting in no overall expression of functional proteins for that MMR gene<sup>29</sup>.

Deleted: -

Deleted: -

Deleted: -

Deleted: -

Deleted: Microsatellite instability

Deleted: hereditary non-polyposis colorectal cancer (

Deleted: )

Deleted: Syndrome

Deleted: Lynch Syndrome

MMR deficiency is not an exclusive characteristic of LS tumours. Approximately 15–20% of sporadic CRCs (mostly colonic cancers) arise due to somatically acquired changes in one of the MMR genes. However, the mechanism of inactivation of MMR in sporadic tumours is generally different from that seen in LS patients. Whereas in LS the defect in MMR function and ensuing mutator phenotype is usually attributable to one germ-line mutation and one acquired mutation or deletion of one of the DNA mismatch repair genes, mostly *MLH1* and *MSH2* in ~90% LS kindreds, the “mutator phenotype” in sporadic colonic cancers is almost entirely caused by hypermethylation of the *MLH1* promoter that results in transcriptional silencing of both copies of *MLH1*<sup>30</sup>. Intriguingly, there is evidence that such tumours develop along a pathway distinct from the classical adenoma–carcinoma sequence<sup>31</sup>. It used to be thought that *MLH1* epigenetic silencing was a mechanism exclusive to sporadic tumours, but a small proportion of LS tumours also inactivate the wild-type allele of *MLH1* via promoter hypermethylation<sup>32, 33</sup>.

Deleted: Lynch Syndrome

Deleted: ,

Deleted: -

Deleted: Lynch Syndrome

Deleted: Lynch Syndrome

Deleted: Lynch Syndrome

Deleted: -

Deleted: Lynch Syndrome

Although up to 15–20% of all colonic cancers exhibit MSI, only a small proportion, of the order of 1–4%, of those will be due to LS. MSI-H appears to be rare in rectal cancers, but when it occurs it usually indicates LS<sup>18</sup>. Likewise, MSI-H is only rarely found in sporadic adenomas, but the adenomas found in LS commonly, but by no means always, have MSI-H. In any case, distinguishing LS from sporadic MMR-deficient tumours requires consideration of a wide range of evidence: from the family history and examination of the tumours for MSI-H and abnormal MMR protein expression, to germ-line mutation analysis.

Deleted: -

Deleted: -

Deleted: Lynch Syndrome

Deleted: however

Deleted: Lynch Syndrome

Deleted: Lynch Syndrome

Deleted: Lynch Syndrome

### *Immunohistochemical detection of MMR proteins*

When LS is suspected, immunohistochemical staining of the MMR proteins can be used to investigate abnormal MMR protein expression that is found in the large majority of LS CRCs, if tumour blocks are available. Abnormal MMR protein expression can be detected in two patterns: either complete loss of expression (when there is no expression of that MMR protein or only expression of a truncated protein to which the antibody does not bind or the mutated protein has lost the epitope recognized by the antibody), or patchy/weak expression (if the mutation generates a prematurely truncated but variably stable protein, or a protein with alterations to the epitope recognized by the antibody, such as may be the case with some missense mutations) (Figure 3). This patchy/weak immunoreactivity may sometimes be seen in the cytoplasm rather than the nucleus, whereas the adjacent normal epithelium or intratumoral activated lymphocytes or stromal cells show the usual pattern of strong nuclear immunopositivity for the MMR protein (due to the expression of the wild-type allele). This suggests that destabilization of the MMR protein complexes has

Deleted: Lynch Syndrome

Deleted: (IHC)

Deleted: Lynch Syndrome

Deleted: recognised

Deleted: recognised

Deleted: Figure

Deleted: staining

Deleted: ,

Deleted: -

Deleted: u

Deleted: destabilisation

occurred and the proteins are no longer bound to the nuclear DNA. Generally, changes in expression of MMR proteins follow the pattern of their heterodimeric complex formation, so that abnormal expression of MSH6 is significantly associated with abnormal MSH2 expression, as stable MSH2-MSH6 heterodimers can not form in the absence of MSH2 and unbound MSH6 appears to be rapidly degraded. Similarly, abnormal expression of PMS2 is significantly associated with abnormal MLH1 expression, as PMS2 forms heterodimers with MLH1.

Deleted: such

Deleted: -

In the clinical setting, if consistent loss of the same MMR protein is observed in different individuals across a family, or in more than one tumour from the same individual, it can be very strong evidence of LS, but it depends on the prior odds of LS being the cause, and the results must be interpreted in the context of the family history, tumour type(s), age of tumour onset and other factors. It is important to realize, however, that a proportion, perhaps 5-20% of HNPCC/Lynch-related tumours do not exhibit any detectable MMR protein expression abnormality on analysis by immunohistochemistry, even though they have lost MMR function, as manifested by MSI-H found on testing tumour DNA. The reasons for this apparent insensitivity are by no means clear. The wide variation reported between different studies is probably a function of differences in technique, such as antigen retrieval, primary antibodies, added to inevitable interobserver variation in interpretation, but it may also be due in part to mutations that functionally inactivate the MMR protein, but which allow its expression as a stable protein with nuclear localization and intact epitope. In this circumstance, where there is a strong family history suggestive of LS, but the MMR immunohistochemistry shows no evidence of abnormality, testing of the tumour DNA for MSI (Figure 1) should be performed using a minimum panel of five

Deleted: Lynch Syndrome

Deleted: Lynch Syndrome

Deleted: /

Deleted: realise

Deleted: -

Deleted: IHC

Deleted: /s

Deleted: -

Deleted: localisation

Deleted: Lynch Syndrome

Deleted: IHC

Deleted: microsatellite instability

Deleted: Figure

Deleted: 5

microsatellite markers (as recommended by the Bethesda guidelines).<sup>34</sup> However, many investigators argue that a greater number of markers should be used and we recommend 10 microsatellite markers<sup>26, 35</sup>. This approach can efficiently direct the search for germ-line mutations to specific MMR genes in appropriately selected families with suspected LS and can be used to interpret any sequence changes found as either pathogenic mutations or harmless polymorphisms.

Deleted: )

Deleted: .

Deleted: Lynch Syndrome

### *Other molecular markers of Lynch syndrome*

A number of other molecular markers are available which can help distinguish between those tumours due to LS and those that have arisen sporadically or due to other predisposition syndromes. A specific mutation, *BRAF* V600E, is found in a proportion of sporadic MSI-H CRCs, but seemingly never (or at least extremely rarely) in CRCs due to LS<sup>36-40</sup>. Hence, finding *BRAF* V600E in a MSI-H (MLH1-negative) CRC is a highly specific marker of sporadic origin (approaching 100% specificity), although the sensitivity is limited (approximately 50%)<sup>41</sup>.

Deleted: Syndrome

Deleted: Lynch Syndrome

Deleted: .

Deleted: from

Deleted: Lynch Syndrome

Deleted: ca.

Deleted: Lynch Syndrome

Formatted: Font: Symbol

Deleted: eta

Deleted: which

A useful complementary test would be one that specifically indicates LS as the cause of a tumour. Activating mutations of  $\beta$ -catenin (*CTNNB1*), especially in exon 3, are observed in that proportion of colorectal tumours that do not apparently harbour mutations in *APC*. Moreover, such mutations are found in tumours with MSI-H due to LS, but not in those with MSI-H of sporadic origin. Although this appears to be a phenomenon specific for LS, it only has low sensitivity (approximately 18%), so is of limited utility<sup>42-45</sup>.

Deleted: Lynch Syndrome

Deleted: which is

Deleted: Lynch Syndrome

Deleted: ca.

### *Genes affected by MMR deficiency*

MMR deficiency within a cell leads to an increased point mutation rate across the whole genome, but with a bias towards mutations in repetitive sequences (microsatellites), particularly mononucleotide repeats. The increase in the mutation rate appears to account for the rapid progression of adenomas to carcinomas in LS and MSI-H sporadic CRCs<sup>46, 47</sup>. Although most microsatellites are in non-coding regions and thus mutations within them would be inconsequential, those repetitive sequences that occur within coding regions of genes of potential significance in growth control make such genes especially vulnerable in cells that have lost MMR function. Such mutations confer a growth advantage and the cells bearing them will be selected for preferential growth during tumour progression. As an alternative to favouring growth directly, such mutations may also favour growth indirectly by allowing escape from immune surveillance mechanisms within the individual. This is demonstrated by an early finding that most, if not all, tumours with MSI-H have lost expression of either  $\beta_2$ -microglobulin (B2M) or HLA class I, thus disabling their ability to present processed abnormal antigens on the cell surface. Tumours with loss of MMR would be expected to express many novel abnormal antigens due to coding region mutations, which, when presented by HLA-I complexed with B2M, would identify such cells to the immune system for destruction. Hence, it is significant that such tumour cells lose antigen presentation by HLA-I/B2M, and moreover by a process involving acquisition of mutations in coding microsatellites in the  $\beta_2$ -microglobulin or HLA-I genes<sup>48-52</sup>. It is estimated that the mutation rate of MMR-defective cancer cells is 100-1000-fold higher than that of normal cells, depending on the amount of carcinogen-induced selective pressure<sup>53, 54</sup>. This phenomenon may account for the acceleration of adenoma-carcinoma progression often seen in LS tumours; however, a rise in the mutation rate in itself does not confer an increase in tumour development rate<sup>55</sup>.

Deleted: Lynch Syndrome

Deleted: Whilst

Deleted: which

Deleted: which

Formatted: Font: Symbol

Deleted: eta-

Formatted: Subscript

Formatted: Font: Symbol

Deleted: eta-

Formatted: Subscript

Deleted: -

Deleted: 1000

Deleted: -

Deleted: Lynch Syndrome

Deleted: .

Genes affected by somatic frameshift mutations that are associated with a defective MMR system in tumours (“MMR targeted genes”) are summarized in Table 1, and these mutations occur at repetitive coding sequences (e.g. (T)<sub>10</sub> tract of 10 thymidine nucleotides in AC1<sub>2</sub> or (A)<sub>8</sub> tract of eight adenine nucleotides in ACVR2).

Deleted: which

Deleted: summarised

Deleted: Table

Deleted: -

Deleted: ,

Deleted: -

Deleted: 8

It is notable that at some loci, e.g. FLT3LG, TEAD2 and BLM, the frequency of mutation is similar in LS and sporadic MSI-H tumours, whereas at others, such as CHK1, B2M and Axin2, the frequencies are different. This is evidence that although LS and sporadic MSI-H tumours that acquire loss of MMR have some overlapping similarities, their underlying pathways of development are distinct. Hence, the validity of combining LS and sporadic MSI+ tumours in studies must be questioned.

Deleted: which

Most repetitive DNA sequences are present in non-coding regions and mutations here are unlikely to confer selectable advantages, However when present in coding regions,

Deleted: ,

Deleted: however

insertion or deletion mutations (typical of unrepaired replication slippage errors occurring in repetitive sequences in MMR-defective cells) result in frameshift mutations that are very likely to introduce a “STOP” codon into the reading frame soon after the frameshift mutation, thus rendering the protein inactive, if not preventing its expression due to nonsense-mediated decay at the mRNA level. The list of genes that are targeted for frameshift mutations in MMR-deficient cells is not confined to those with a coding repetitive sequence. As has been discussed, loss of MMR predisposes a cell to base mismatch mutations all over the genome; for example, *BRAF* is frequently mutated (usually the V600E mutation) in sporadic MSI-H CRCs, but not in LS tumours. It has been suggested that this BRAF V600E mutation may associate more with a tendency to acquire the CpG island methylator

Deleted: ,

Deleted: Lynch Syndrome

phenotype that inactivates *MLH1* via methylation of the CpG islands in the *MLH1* promoter<sup>24, 39</sup>. In contrast, *PTEN* with a repetitive sequence in the coding region is found to be mutated in both MSI-H and MSS tumours<sup>60</sup>, suggesting that there is an intrinsic selective pressure for mutation at this gene locus, which is not necessarily driven by MMR deficiency.

Deleted: (CIMP)

Deleted: ,

### *Prognostic significance of MMR deficiency in CRC*

The selectable advantage conferred on tumour cells by the acquisition of MMR deficiency is contentious and may in part relate to changes in susceptibility to apoptosis<sup>10, 11, 61, 62</sup>. It was noted even at the initial description of MSI, that those patients with MSI-H tumours appeared to have better survival rates than those with MSS tumours<sup>21</sup>. Since then, there have been many studies addressing the prognostic significance of MSI-H in CRC, with most of them agreeing with the initial findings<sup>63-66</sup>, particularly in young patients<sup>67</sup>, and a few studies failing to identify MSI status as an independent prognostic factor<sup>68, 69</sup>. In apparent support of the prognostic value of the MSI status is the high prevalence of activated intraepithelial cytotoxic T lymphocytes and increased neoplastic cell apoptosis in MSI-H tumours<sup>63, 65</sup>. The former characteristic could be attributable to the inherent capacity of MSI-H tumours to produce new immunogenic epitopes (if [B2M](#) and HLA class I loci are intact and these novel antigens can be presented), and this could explain why some patients with MSI-H tumours have a particularly favourable clinical outcome following effective antitumour immune responses.

Deleted: beta-2-microglobulin

Deleted: ,

Deleted: -



The predictive significance of MSI-H has also been assessed in relation to the selection of CRC patients to receive adjuvant chemotherapy. The main correlation that has been shown is the recognition of 5-fluorouracil (5FU)-induced DNA lesions by the intact MMR system<sup>70-72</sup> resulting in MMR-deficient cells being more resistant to 5-FU than MMR-proficient cells. Interestingly, when the MMR deficiency was due to MLH1 hypermethylation the cells regained their sensitivity to 5-FU upon MLH1 demethylation<sup>73</sup>. However, a clear-cut role for MSI-H as a predictor of response to chemotherapy is still controversial<sup>74-76</sup>.

Deleted: -

The molecular basis underlying MSI is different from CIN and so the clinical, histopathological and genetic features accompanying them are not unexpectedly different. Interestingly, there is a statistically significant negative association between MSI-H and mutations in *APC* and *TP53* genes that have previously been associated with DNA aneuploidy<sup>77-79</sup>. This observation could also partly account for the less aggressive phenotype of some MSI-H tumours<sup>76</sup>.

Deleted: which

Although MSI testing can be carried out relatively easily in molecular diagnostic laboratories, there are doubts over the clinical utility of MSI-H as a prognostic marker in sporadic CRC<sup>80, 81</sup>. An important factor in the failure to reach a consensus is the variation in the type and number of microsatellite markers that have been assessed by different studies. A large meta-analysis<sup>82</sup> with  $\geq 7000$  patients has shown that there is a clear correlation between MSI-H tumours and improved overall survival. However, this study was performed retrospectively and thus could be liable to various confounding factors. It is also possible that differences in the underlying molecular pathways of LS and sporadic MSI-H tumours, as shown by the mutation frequency data in Table 1, also play a part, and thus there may be important differences between LS and sporadic MSI-H tumours that are obscured by combination of their data.

Deleted: ,

Deleted: more than

Deleted: quite

Deleted: Table

Deleted: s

Deleted: which

### *Conclusion and future perspectives*

Understanding the role of genomic instability in cancer initiation and progression is growing with better insight into the biological and clinical implications of stratifying CRCs according to the presence or absence of MMR deficiency. It is becoming increasingly clear that the underlying genotypic background of each tumour governs its proliferative, invasive and metastatic capacities as well its response to chemotherapy. Analysis of MMR deficiency by the combination of MMR protein immunohistochemistry and detection of MSI status is warranted as a screening approach for [LS](#) and could provide useful prognostic information in patients with either hereditary or sporadic MMR-deficient tumours.

Deleted: -

Deleted: Lynch Syndrome

The mutational targets in MMR-deficient tumours are not random, in that mutations preferentially occur in repetitive sequences, but nonetheless, there is no *a priori* favouring of mutation site. However, those mutations [that](#) are observed are those [that](#) are positively selected, presumably on the basis that they confer a Darwinian selectable advantage via key regulatory roles in cell growth, survival and escape from immune surveillance. If this hypothesis is correct, identification of the genes that become mutated as a result of MMR deficiency is likely to be valuable in developing new therapeutics, and could form the basis of [personalized](#) selection of cancer-related therapy in the future. In any case, conclusive assessment of the prognostic significance of MMR deficiency would require large prospective studies, using standardized MSI testing and MMR immunohistochemistry criteria, and rigorous distinction and separation of MSI-H tumours due to LS from those that have arisen sporadically. There would also have to be independence from confounding factors such as the grade and stage of the tumour, age of the patient and treatment.

Deleted: .

Formatted: Font: Italic

Deleted: which

Deleted: which

Deleted: -

Deleted: -

Deleted: personalised

Deleted: -

**Figure 1.** Examples of microsatellite instability observed in colorectal cancers (courtesy of [J. Myring](#) and [R. Hussein](#), Institute of Medical Genetics, Cardiff, [UK](#)).

Polymerase chain reaction (PCR)-amplified microsatellites are analysed in a fluorescent DNA analyser (ABI 3100), in which fluorescently labelled DNA molecules are separated by size using capillary gel electrophoresis. In each frame the PCR products from the cancer are labelled in red, with corresponding normal tissue (or blood) labelled in green. Each peak represents a DNA molecule of a specific length. Although individual alleles in the DNA are of a specific length, say 128 bp, the PCR process introduces stutters and hence a spread of peaks around a modal size. Microsatellite instability at a particular marker is identified by the presence of either new peaks (modal peak with adjacent stutter peaks) or a significant change in height of peaks—both are indicated by short black arrows. Frames a–c are polyadenine mononucleotide repeats of the form (A)<sub>n</sub>, whereas frames d–f are dinucleotide repeats of the form (CA)<sub>n</sub>. BAT25 is located in intron 16 of the oncogene c-kit at chromosome 4q12, BAT40 is in intron 2 of the 3-beta-hydroxysteroid dehydrogenase gene HSD3 at 1p11, ACTC is in intron IV of the cardiac alpha actin gene ACTC1 at 15q14, D5S346 is close to APC at 5q22, and D5S406 is at 5p15<sup>26, 84</sup>.

Deleted: Figure Legends

Deleted: Figure

Formatted: Line spacing: Double

Deleted: Jenny

Deleted: Rana

Deleted: PCR

Deleted: 128

Deleted: –

Deleted:

Deleted:

Deleted:

Deleted:

**Figure 2.** Mismatch repair pathways in eukaryotes. The MutS and MutL heterodimer complexes can vary in composition, with MutL complexes containing the MLH1 protein plus one of the other four binding partners (PMS2, MLH3, PMS1 or MLH2); similarly, MutS complexes contain the MSH2 protein plus one of the other five binding partners (usually either MSH6 or MSH3). MutS and MutL heterodimers interact to form larger functional heterocomplexes that contribute to the repair of different types of DNA alteration: base mismatches or single nucleotide insertion/deletion loops (first “Mismatch” column); single-strand insertion-deletion loops of two to eight nucleotides (second “Insertion/deletion loops” column); DNA double-strand breaks (third column); and Holliday junction resolution (fourth column). Binding of the MutS heterodimer to mismatched DNA triggers the ATP-dependent interaction of the second MutL heterodimer that leads to strand discrimination and error removal. The process of excision of the DNA strand containing the error and re-synthesis of correctly matched DNA is facilitated by other factors including exonuclease 1 (EXO1), the proliferating cell nuclear antigen (PCNA), replication factor C (RF-C), and DNA polymerases  $\delta$  and  $\epsilon$ , while the repaired strand is re-ligated with DNA ligase I. (Figure adapted with modifications from Augusto-Pinto *et al.*<sup>83</sup> and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways annotation).

**Deleted:** Figure  
**Formatted:** Line spacing: Double

**Deleted:** 4  
**Deleted:** ),  
**Deleted:** 5

**Deleted:**  
**Deleted:**

**Deleted:** -  
**Deleted:** 2-8

**Deleted:** et  
**Formatted:** Font: Italic  
**Deleted:** *al*  
**Deleted:** Figure  
**Formatted:** Line spacing: Double  
**Deleted:** MMR  
**Deleted:** (  
**Deleted:** -  
**Deleted:** )  
**Formatted:** Font: Not Bold  
**Deleted:** immunostaining  
**Formatted:** Font: Not Bold  
**Deleted:** high  
**Deleted:** (x400 magnification)  
**Deleted:** IHC  
**Deleted:** positive  
**Deleted:** staining  
**Formatted:** Font: Bold

**Figure 3.** Examples of abnormal mismatch repair protein immunohistochemistry observed in colorectal cancers. **a-d.** Loss of MSH2 immunoreactivity in a colonic cancer with secondary loss of its binding partner MSH6. Panels show high-power photomicrographs of immunohistochemistry of nuclear MLH1 immunopositivity (**a**)

and nuclear PMS2 [immunopositivity](#) (c), but nuclear MSH2 [immunonegativity](#) (b) and nuclear MSH6 [immunonegativity](#) (d), although some patchy weak cytoplasmic MSH6 [reactivity](#) can be seen in a few tumour cells, with retention of strong nuclear [reactivity](#) of the lymphoid and stromal cells as internal positive controls in (b) and (d).

**e– h.** Patchy/weak [immunoreactivity](#) of MLH1 in a caecal cancer with secondary loss of its binding partner PMS2. Panels show [medium](#)-power photomicrographs of [immunohistochemistry](#) of nuclear MSH2 [immunopositivity](#) (f) and nuclear MSH6 [immunopositivity](#) (h), but patchy/weak nuclear MLH1 [positivity](#) (e) and nuclear PMS2 [immunonegativity](#) (g), with retention of strong nuclear [reactivity](#) of the lymphoid and stromal cells as internal positive controls in (e) and (g).

## References

1. Cook PJ, Doll R, Fellingham SA. A mathematical model for the age distribution of cancer in man. *Int. J. Cancer* 1969; **4**: 93–112.
2. Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000; **100**: 57–70.
3. Wood LD, Parsons DW, Jones S *et al.* The genomic landscapes of human breast and colorectal cancers. *Science* 2007; **318**(5853): 1108–1113.
4. Sjoblom T, Jones S, Wood LD *et al.* The consensus coding sequences of human breast and colorectal cancers. *Science* 2006; **314**(5797): 268–274.
5. Lengauer C, Kinzler KW, Vogelstein B. Genetic instability in colorectal cancers. *Nature* 1997; **386**(6625): 623–627.
6. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. *Nature* 1998; **396**(6712): 643–649.

Deleted: positive ...staining ... [1]

Deleted: negative ...staining ... [2]

Formatted: Font: Bold

Formatted: Font: Bold

Deleted: negative ...staining ... [3]

Formatted: Font: Bold

Deleted: staining ...staining ... [4]

Formatted: ... [5]

Deleted: (... ..) ... [6]

Formatted: Font: Not Bold

Deleted: immunostaining

Formatted: Font: Not Bold

Deleted: medium ... (x200 magnification) ... IHC ... positive ... [7]

Formatted: Font: Bold

Deleted: positive ...staining ... [8]

Formatted: Font: Bold

Deleted: staining

Formatted: Font: Bold

Deleted: negative ...staining ... [9]

Formatted: Font: Bold

Deleted: staining

Formatted: ... [10]

Formatted: Font: Not Italic

Formatted: Line spacing: Double

Deleted: Jan 15... (1); ... [11]

Formatted: Font: Bold

Deleted: -

Deleted: Jan 7... (1); ... [12]

Formatted: Font: Bold

Deleted: -

Deleted: et ... Nov 16...; ... [13]

Deleted: et ... Oct 13...; ... [14]

Deleted: Apr 10...; ... [15]

Deleted: Dec 17...; ... [16]

<p>7. Holt KE, Parkhill J, Mazzoni CJ <i>et al.</i> High-throughput sequencing provides insights into genome variation and evolution in Salmonella Typhi. <i>Nat. Genet.</i> 2008; <b>40</b>: 987–993.</p>	<p>Deleted: <i>et</i></p> <p>Deleted: Aug</p> <p>Deleted: (8);</p> <p>Deleted: -</p> <p>Deleted: Jun</p>
<p>8. Holt RA, Jones SJ. The new paradigm of flow cell sequencing. <i>Genome Res.</i> 2008; <b>18</b>: 839–846.</p>	<p>Deleted: (6);</p> <p>Deleted: -</p> <p>Deleted: <i>et</i></p>
<p>9. Zhang K, Martiny AC, Reppas NB <i>et al.</i> Sequencing genomes from single cells by polymerase cloning. <i>Nat. Biotechnol.</i> 2006; <b>24</b>: 680–686.</p>	<p>Deleted: Jun</p> <p>Deleted: (6);</p> <p>Deleted: -</p>
<p>10. Tomlinson IP, Novelli MR, Bodmer WF. The mutation rate and cancer. <i>Proc. Natl Acad. Sci. USA</i> 1996; <b>93</b>: 14800–14803.</p>	<p>Deleted:</p> <p>Deleted:</p> <p>Deleted: Dec 10</p>
<p>11. Toft NJ, Curtis LJ, Sansom OJ <i>et al.</i> Heterozygosity for p53 promotes microsatellite instability and tumorigenesis on a Msh2 deficient background. <i>Oncogene</i> 2002; <b>21</b>: 6299–6306.</p>	<p>Deleted: (25);</p> <p>Deleted: -</p> <p>Deleted: <i>et</i></p> <p>Deleted: Sep 12</p>
<p>12. Toft NJ, Winton DJ, Kelly J <i>et al.</i> Msh2 status modulates both apoptosis and mutation frequency in the murine small intestine. <i>Proc. Natl Acad. Sci. USA</i> 1999; <b>96</b>: 3911–3915.</p>	<p>Deleted: (41);</p> <p>Deleted: -</p> <p>Deleted: <i>et</i></p> <p>Deleted:</p> <p>Deleted:</p> <p>Deleted: Mar 30</p>
<p>13. Tomlinson IP, Bodmer WF. Failure of programmed cell death and differentiation as causes of tumors: some simple mathematical models. <i>Proc. Natl Acad. Sci. USA</i> 1995; <b>92</b>: 11130–11134.</p>	<p>Deleted: (7);</p> <p>Deleted: -</p> <p>Deleted:</p> <p>Deleted:</p> <p>Deleted: Nov 21</p>
<p>14. Daya-Grosjean L, Sarasin A. The role of UV induced lesions in skin carcinogenesis: an overview of oncogene and tumor suppressor gene modifications in xeroderma pigmentosum skin tumors. <i>Mutat. Res.</i> 2005; <b>571</b>: 43–56.</p>	<p>Deleted: (24);</p> <p>Deleted: -</p> <p>Deleted: Apr 1</p> <p>Deleted: (1-2);</p> <p>Deleted: -</p>
<p>15. de Vos M, Hayward B, Bonthron DT, Sheridan E. Phenotype associated with recessively inherited mutations in DNA mismatch repair (MMR) genes. <i>Biochem. Soc. Trans.</i> 2005; <b>33</b>: 718–720.</p>	<p>Deleted: Aug</p> <p>Deleted: (Pt 4);</p> <p>Deleted: -</p>

16. Chung DC, Rustgi AK. DNA mismatch repair and cancer. *Gastroenterology* 1995; **109**: 1685–1699.
17. Vasen HF, Moslein G, Alonso A *et al.* Guidelines for the clinical management of Lynch Syndrome (hereditary non-polyposis cancer). *J. Med. Genet.* 2007; **44**: 353–362.
18. Nilbert M, Planck M, Fernebro E, Borg A, Johnson A. Microsatellite instability is rare in rectal carcinomas and signifies hereditary cancer. *Eur. J. Cancer* 1999; **35**: 942–945.
19. Sieber OM, Heinimann K, Tomlinson IP. Genomic instability—the engine of tumorigenesis? *Nat. Rev. Cancer* 2003; **3**: 701–708.
20. Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M. Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 1993; **363** (6429): 558–561.
21. Thibodeau SN, Bren G, Schaid D. Microsatellite instability in cancer of the proximal colon. *Science* 1993; **260** (5109): 816–819.
22. Laiho P, Launonen V, Lahermo P *et al.* Low-level microsatellite instability in most colorectal carcinomas. *Cancer Res.* 2002; **62**: 1166–1170.
23. Halford S, Sasieni P, Rowan A *et al.* Low-level microsatellite instability occurs in most colorectal cancers and is a nonrandomly distributed quantitative trait. *Cancer Res.* 2002; **62**: 53–57.
24. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* 2007; **50**: 113–130.
25. Jass JR, Iino H, Ruzskiewicz A *et al.* Neoplastic progression occurs through mutator pathways in hyperplastic polyposis of the colorectum. *Gut* 2000; **47**: 43–49.
- Deleted: Nov
- Deleted: (5);
- Deleted: -
- Deleted: *et*
- Deleted: Jun
- Deleted: (6);
- Deleted: -
- Deleted: Jun
- Deleted: (6);
- Deleted: -
- Deleted: --
- Deleted: Sep
- Deleted: (9);
- Deleted: -
- Deleted: Jun 10
- Deleted: ;
- Deleted: -
- Deleted: May 7
- Deleted: ;
- Deleted: -
- Deleted: *et*
- Deleted: Feb 15
- Deleted: (4);
- Deleted: -
- Deleted: *et*
- Deleted: Jan 1
- Deleted: (1);
- Deleted: -
- Deleted: Jan
- Deleted: (1);
- Deleted: -
- Deleted: *et*
- Deleted: Jul
- Deleted: (1);
- Deleted: -

26. Frayling IM, Happerfield L, Mattocks C, Oakhill K, Arends MJ. Application of molecular diagnostics to hereditary

nonpolyposis colorectal cancer. In [Coleman WB, Tsongalis GJ eds. \*Molecular diagnostics for the clinical laboratorian\*, 2nd edn. Totowa, NJ: Humana Press Inc., 2005; 375–392.](#)

Deleted: T

Deleted: ,

Deleted: .

27. Detloff P, White MA, Petes TD. Analysis of a gene conversion gradient at the HIS4 locus in *Saccharomyces cerevisiae*. *Genetics* 1992; **132**: 113–123.

Formatted: Font: Italic

Deleted: Sep

Deleted: (1);

Deleted: -

Formatted: Font: Bold

Deleted: -

28. Boland CR. Hereditary nonpolyposis colorectal cancer. *The genetic basis of human cancer*. New York: McGraw-Hill, 1998; 333–346.

Deleted: *et*

29. Parsons R, Li GM, Longley MJ *et al*. Hypermutability and mismatch repair deficiency in RER+ tumor cells. *Cell* 1993; **75**: 1227–1236.

Deleted: Dec 17

Deleted: (6);

Deleted: -

30. Cunningham JM, Christensen ER, Tester DJ *et al*. Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. *Cancer Res.* 1998;

Deleted: *et*

Deleted: Aug 1

**58**: 3455–3460.

Deleted: (15);

Deleted: -

31. Hawkins NJ, Ward RL. Sporadic colorectal cancers with microsatellite instability and their possible origin in hyperplastic polyps and serrated adenomas. *J. Natl Cancer Inst.* 2001; **93**: 1307–1313.

Deleted: Sep 5

Deleted: (17);

Deleted: -

32. Hitchins M, Williams R, Cheong K *et al*. MLH1 germline epimutations as a factor in hereditary nonpolyposis colorectal cancer. *Gastroenterology* 2005; **129**: 1392–1399.

Deleted: *et*

Deleted: Nov

Deleted: (5);

Deleted: -

33. Suter CM, Martin DI, Ward RL. Germline epimutation of MLH1 in individuals with multiple cancers. *Nat. Genet.* 2004; **36**: 497–501.

Deleted: May

Deleted: (5);

Deleted: -

34. Umar A, Boland CR, Terdiman JP *et al*. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch Syndrome) and microsatellite instability. *J. Natl Cancer Inst.* 2004; **96**: 261–268.

Deleted: *et*

Deleted: Feb 18

Deleted: (4);

Deleted: -



35. Arends MJ FI. The genetics of colorectal cancer: mismatch repair deficiency in hereditary and sporadic colorectal cancer. In Cunningham D, [Topham C](#), Miles A eds. *Key advances in the effective management of colorectal cancer*. London: Aesculapius

Medical Press, 2004; 25–40.

Deleted: pp

36. Miyaki M, Iijima T, Kimura J *et al*. Frequent mutation of beta-catenin and APC genes in primary colorectal tumors from patients with hereditary nonpolyposis

colorectal cancer. *Cancer Res*. 1999; **59**: 4506–4509.

Deleted: et

Deleted: Sep 15

Deleted: (18);

Formatted: Font: Bold

Deleted: -

Deleted: et

Deleted: Sep

37. Domingo E, Laiho P, Ollikainen M *et al*. BRAF screening as a low-cost effective strategy for simplifying HNPCC genetic testing. *J. Med. Genet*. 2004; **41**: 664–668.

38. Domingo E, Niessen RC, Oliveira C *et al*. BRAF-V600E is not involved in the colorectal tumorigenesis of HNPCC in patients with functional MLH1 and MSH2

genes. *Oncogene* 2005; **24**: 3995–3998.

Deleted: (9);

Deleted: -

Deleted: et

Deleted: Jun 2

Deleted: (24);

Deleted: -

39. Deng G, Bell I, Crawley S *et al*. BRAF mutation is frequently present in sporadic colorectal cancer with methylated hMLH1, but not in hereditary nonpolyposis

colorectal cancer. *Clin. Cancer Res*. 2004; **10**: 191–195.

Deleted: et

Deleted: Jan 1

Deleted: (1 Pt 1);

Deleted: -

40. McGivern A, Wynter CV, Whitehall VL *et al*. Promoter hypermethylation frequency and BRAF mutations distinguish hereditary non-polyposis colon cancer

from sporadic MSI-H colon cancer. *Fam. Cancer* 2004; **3**: 101–107.

Deleted: et

Deleted: (2);

Deleted: -

Deleted: et

41. Loughrey MB, Waring PM, Tan A *et al*. Incorporation of somatic BRAF mutation testing into an algorithm for the investigation of hereditary non-polyposis colorectal

cancer. *Fam. Cancer* 2007; **6**: 301–310.

Deleted: (3);

Deleted: -

42. Akiyama Y, Nagasaki H, Yagi KO, Nomizu T, Yuasa Y. Beta-catenin and adenomatous polyposis coli (APC) mutations in adenomas from hereditary non-polyposis colorectal cancer patients. *Cancer Lett*. 2000; **157**: 185–191.

Deleted: Sep 1

Deleted: (2);

Deleted: -

<p>43. Shitoh K, Furukawa T, Kojima M <i>et al.</i> Frequent activation of the beta-catenin-Tcf signaling pathway in nonfamilial colorectal carcinomas with microsatellite instability. <i>Genes Chromosomes Cancer</i> 2001; <b>30</b>: 32–37.</p>	<p>Deleted: <i>et</i></p>
<p>44. Fukushima H, Yamamoto H, Itoh F <i>et al.</i> Frequent alterations of the beta-catenin and TCF-4 genes, but not of the APC gene, in colon cancers with high-frequency microsatellite instability. <i>J. Exp. Clin. Cancer Res.</i> 2001; <b>20</b>: 553–559.</p>	<p>Deleted: Jan Deleted: (1); Deleted: - Deleted: <i>et</i></p>
<p>45. Johnson V, Volikos E, Halford SE <i>et al.</i> Exon 3 beta-catenin mutations are specifically associated with colorectal carcinomas in hereditary non-polyposis colorectal cancer syndrome. <i>Gut</i> 2005; <b>54</b>: 264–267.</p>	<p>Deleted: Dec Deleted: (4); Deleted: - Deleted: <i>et</i></p>
<p>46. Tsao JL, Yatabe Y, Salovaara R <i>et al.</i> Genetic reconstruction of individual colorectal tumor histories. <i>Proc. Natl Acad. Sci. USA</i> 2000; <b>97</b>: 1236–1241.</p>	<p>Deleted: Feb Deleted: (2); Deleted: - Deleted: <i>et</i></p>
<p>47. Aaltonen LA, Peltomaki P, Leach FS <i>et al.</i> Clues to the pathogenesis of familial colorectal cancer. <i>Science</i> 1993; <b>260</b> (5109): 812–816.</p>	<p>Deleted: Deleted: Deleted: Feb 1 Deleted: (3); Deleted: - Deleted: <i>et</i></p>
<p>48. Kaklamani L, Gatter KC, Hill AB <i>et al.</i> Loss of HLA class-I alleles, heavy chains and beta 2-microglobulin in colorectal cancer. <i>Int. J. Cancer</i> 1992; <b>51</b>: 379–385.</p>	<p>Deleted: May 7 Deleted: ; Deleted: - Deleted: <i>et</i></p>
<p>49. Bicknell DC, Rowan A, Bodmer WF. Beta 2-microglobulin gene mutations: a study of established colorectal cell lines and fresh tumors. <i>Proc. Natl Acad. Sci. USA</i> 1994; <b>91</b>: 4751–4755.</p>	<p>Deleted: May 28 Deleted: (3); Deleted: - Deleted: Deleted:</p>
<p>50. Bicknell DC, Kaklamani L, Hampson R, Bodmer WF, Karran P. Selection for beta 2-microglobulin mutation in mismatch repair-defective colorectal carcinomas. <i>Curr. Biol.</i> 1996; <b>6</b>: 1695–1697.</p>	<p>Deleted: May 24 Deleted: (11); Deleted: - Deleted: Dec 1 Deleted: (12); Deleted: - Deleted: <i>et</i></p>
<p>51. Dierssen JW, de Miranda NF, Ferrone S <i>et al.</i> HNPCC versus sporadic microsatellite-unstable colon cancers follow different routes toward loss of HLA class I expression. <i>BMC Cancer</i> 2007; <b>7</b>: 33.</p>	<p>Deleted: ;</p>

52. Kloor M, Michel S, Buckowitz B *et al.* Beta2-microglobulin mutations in microsatellite unstable colorectal tumors. *Int. J. Cancer* 2007; **121**: 454–458.
53. Bhattacharyya NP, Skandalis A, Ganesh A, Groden J, Meuth M. Mutator phenotypes in human colorectal carcinoma cell lines. *Proc. Natl Acad. Sci. USA* 1994; **91**: 6319–6323.
54. Glaab WE, Risinger JI, Umar A, Barrett JC, Kunkel TA, Tindall KR. Resistance to 6-thioguanine in mismatch repair-deficient human cancer cell lines correlates with an increase in induced mutations at the HPRT locus. *Carcinogenesis* 1998; **19**: 1931–1937.
55. Shibata D. Molecular tumour clocks and colorectal cancer: seeing the unseen. *Pathology* 2002; **34**: 534–540.
56. Woerner SM, Kloor M, Mueller A *et al.* Microsatellite instability of selective target genes in HNPCC-associated colon adenomas. *Oncogene* 2005; **24**: 2525–2535.
57. Woerner SM, Benner A, Sutter C *et al.* Pathogenesis of DNA repair-deficient cancers: a statistical meta-analysis of putative Real Common Target genes. *Oncogene* 2003; **22**: 2226–2235.
58. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology* 2008; **135**: 1079–1099.
59. Huang J, Papadopoulos N, McKinley AJ *et al.* APC mutations in colorectal tumors with mismatch repair deficiency. *Proc. Natl Acad. Sci. USA* 1996; **93**: 9049–9054.
60. Nassif NT, Lobo GP, Wu X *et al.* PTEN mutations are common in sporadic microsatellite stable colorectal cancer. *Oncogene* 2004; **23**: 617–628.
61. Fishel R. The selection for mismatch repair defects in hereditary nonpolyposis colorectal cancer: revising the mutator hypothesis. *Cancer Res.* 2001; **61**: 7369–7374.

Deleted: *et*

Deleted: Jul 15

Deleted: (2);

Deleted: -

Deleted:

Deleted:

Deleted: Jul 5

Deleted: (14);

Deleted: -

Deleted: Nov

Deleted: (11);

Deleted: -

Deleted: Dec

Deleted: (6);

Deleted: -

Deleted: *et*

Deleted: Apr 7

Deleted: (15);

Deleted: -

Deleted: *et*

Deleted: Apr 17

Deleted: (15);

Deleted: -

Deleted: Sep 3.

Deleted: *et*

Deleted:

Deleted:

Deleted: Aug 20

Deleted: (17);

Deleted: -

Deleted: *et*

Deleted: Jan 15

Deleted: (2);

Deleted: -

Deleted: Oct 15

Deleted: (20);

Deleted: -

62. Tomlinson I, Bodmer W. Selection, the mutation rate and cancer: ensuring that the tail does not wag the dog. <i>Nat. Med.</i> 1999; <b>5</b> : 11–12.	Deleted: Jan
	Deleted: (1);
	Deleted: -
63. Benatti P, Gafa R, Barana D <i>et al.</i> Microsatellite instability and colorectal cancer prognosis. <i>Clin. Cancer Res.</i> 2005; <b>11</b> : 8332–8340.	Deleted: <i>et</i>
	Deleted: Dec 1
	Deleted: (23);
64. Guidoboni M, Gafa R, Viel A <i>et al.</i> Microsatellite instability and high content of activated cytotoxic lymphocytes identify colon cancer patients with a favorable prognosis. <i>Am. J. Pathol.</i> 2001; <b>159</b> : 297–304.	Deleted: -
	Deleted: <i>et</i>
65. Samowitz WS, Curtin K, Ma KN <i>et al.</i> Microsatellite instability in sporadic colon cancer is associated with an improved prognosis at the population level. <i>Cancer Epidemiol. Biomarkers Prev.</i> 2001; <b>10</b> : 917–923.	Deleted: Jul
	Deleted: (1);
	Deleted: -
	Deleted: <i>et</i>
66. Watanabe Y, Nakajima H, Nozaki K <i>et al.</i> Clinicopathologic and immunohistochemical features and microsatellite status of endometrial cancer of the uterine isthmus. <i>Int. J. Gynecol. Pathol.</i> 2001; <b>20</b> : 368–373.	Deleted: Sep
	Deleted: (9);
	Deleted: -
	Deleted: <i>et</i>
67. Gryfe R, Kim H, Hsieh ET <i>et al.</i> Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. <i>N. Engl. J. Med.</i> 2000; <b>342</b> : 69–77.	Deleted: Oct
	Deleted: (4);
	Deleted: -
	Deleted: <i>et</i>
68. Ward RL, Cheong K, Ku SL, Meagher A, O'Connor T, Hawkins NJ. Adverse prognostic effect of methylation in colorectal cancer is reversed by microsatellite instability. <i>J. Clin. Oncol.</i> 2003; <b>21</b> : 3729–3736.	Deleted: Jan 13
	Deleted: (2);
	Deleted: -
69. Salahshor S, Kressner U, Fischer H <i>et al.</i> Microsatellite instability in sporadic colorectal cancer is not an independent prognostic factor. <i>Br. J. Cancer</i> 1999; <b>81</b> : 190–193.	Deleted: Oct 15
	Deleted: (20);
	Deleted: -
	Deleted: <i>et</i>
	Deleted: Sep
70. Carethers JM, Chauhan DP, Fink D <i>et al.</i> Mismatch repair proficiency and <i>in vitro</i> response to 5-fluorouracil. <i>Gastroenterology</i> 1999; <b>117</b> : 123–131.	Deleted: (2);
	Deleted: -
	Deleted: <i>et</i>
	Formatted: Font: Italic
	Deleted: Jul
	Deleted: (1);
	Deleted: -

71. Tajima A, Hess MT, Cabrera BL, Kolodner RD, Carethers JM. The mismatch repair complex hMutS alpha recognizes 5-fluorouracil-modified DNA: implications

for chemosensitivity and resistance. *Gastroenterology* 2004; **127**: 1678–1684.

Deleted: Dec

Deleted: (6);

Deleted: -

72. Meyers M, Wagner MW, Mazurek A, Schmutte C, Fishel R, Boothman DA. DNA mismatch repair-dependent response to fluoropyrimidine-generated damage. *J. Biol. Chem.* 2005; **280**: 5516–5526.

Deleted: Feb 18

Deleted: (7);

Deleted: -

73. Arnold CN, Goel A, Boland CR. Role of hMLH1 promoter hypermethylation in drug resistance to 5-fluorouracil in colorectal cancer cell lines. *Int. J. Cancer* 2003;

Deleted: Aug 10

**106**: 66–73.

Deleted: (1);

Deleted: -

74. Hemminki A, Mecklin JP, Jarvinen H, Aaltonen LA, Joensuu H. Microsatellite instability is a favorable prognostic indicator in patients with colorectal cancer receiving chemotherapy. *Gastroenterology* 2000; **119**: 921–928.

Deleted: Oct

Deleted: (4);

Deleted: -

75. Halling KC, French AJ, McDonnell SK *et al.* Microsatellite instability and 8p

Deleted: *et*

allelic imbalance in stage B2 and C colorectal cancers. *J. Natl Cancer Inst.* 1999; **91**: 1295–1303.

Deleted: Aug 4

Deleted: (15);

Deleted: -

Deleted: *et*

76. Elsaleh H, Powell B, Soontrapornchai P *et al.* p53 gene mutation, microsatellite instability and adjuvant chemotherapy: impact on survival of 388 patients with Dukes' C colon carcinoma. *Oncology* 2000; **58**: 52–59.

Deleted: (1);

Deleted: -

Deleted: *et*

77. Meling GI, Lothe RA, Borresen AL *et al.* The TP53 tumour suppressor gene in colorectal carcinomas. II. Relation to DNA ploidy pattern and clinicopathological variables. *Br. J. Cancer* 1993; **67**: 93–98.

Deleted: Jan

Deleted: (1);

Deleted: -

78. Salahshor S, Kressner U, Pahlman L, Glimelius B, Lindmark G, Lindblom A.

Colorectal cancer with and without microsatellite instability involves different genes.

*Genes Chromosomes Cancer* 1999; **26**: 247–252.

Deleted: Nov

Deleted: (3);

Deleted: -

79. Fodde R, Kuipers J, Rosenberg C *et al.* Mutations in the APC tumour suppressor gene cause chromosomal instability. *Nat. Cell Biol.* 2001; **3**: 433–438.

80. Boland CR. Clinical uses of microsatellite instability testing in colorectal cancer: an ongoing challenge. *J. Clin. Oncol.* 2007; **25**: 754–756.

81. Kim GP, Colangelo LH, Wieand HS *et al.* Prognostic and predictive roles of high-degree microsatellite instability in colon cancer: a National Cancer Institute-National

Deleted: *et*

Deleted:

Deleted: Apr

Deleted: (4);

Deleted: -

Deleted: Mar 1

Deleted: (7);

Deleted: -

Deleted: *et*

Surgical Adjuvant Breast and Bowel Project Collaborative Study. *J. Clin. Oncol.* 2007; **25**: 767–772.

82. Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. *J. Clin. Oncol.* 2005; **23**: 609–618.

83. Augusto-Pinto L, da Silva CG, Lopes Dde O, Machado-Silva A, Machado CR. *Escherichia coli* as a model system to study DNA repair genes of eukaryotic organisms. *Genet. Mol. Res.* 2003; **2**: 77–91.

Deleted: Mar 1

Deleted: (7);

Deleted: -

Deleted: Jan 20

Deleted: (3);

Deleted: -

Formatted: Font: Italic

Deleted: (1);

Deleted: -

84. Sutter C, Gebert J, Bischoff P, Herfarth C, von Knebel Doeberitz M. Molecular screening of potential HNPCC patients using a multiplex microsatellite PCR system. *Mol. Cell. Probes* 1999; **13**: 157–165.

Deleted: Apr

Deleted: (2);

Deleted: -

**Table 1.** Mutation frequency of “MMR targeted genes” in MMR-deficient CRCs\*

<i>Gene</i>	<i>Chromosome</i> <sup>†</sup>	<i>Mutated target sequence</i>	<i>MSI-H (LS), %</i>	<i>MSI-H (sporadic), %</i>
<a href="#">AC1</a>	<a href="#">4p16.1</a>	<a href="#">(T)<sub>10</sub></a>	<a href="#">67</a>	<a href="#">=</a>
<a href="#">ACVR2</a>	<a href="#">2q22.3-q23.1</a>	<a href="#">(A)<sub>8</sub></a>	<a href="#">72</a>	<a href="#">83</a>
<a href="#">AIM2</a>	<a href="#">1q23.2</a>	<a href="#">(A)<sub>10</sub></a>	<a href="#">56</a>	<a href="#">45</a>
<a href="#">APC</a>	<a href="#">5q21-q22</a>	<a href="#">variable<sup>‡</sup></a>	<a href="#">56</a>	<a href="#">56</a>

Formatted: Font: Not Bold

Formatted: Not Superscript/ Subscript

<u>Axin2</u>	<u>17q24.1</u>	<u>(G)<sub>7</sub></u>	<u>20</u>	<u>11</u>
<u>B2M</u>	<u>15q21.1</u>	<u>(CT)<sub>4</sub> / (A)<sub>5</sub></u>	<u>36.4</u>	<u>15.4</u>
<u>BAX</u>	<u>19q13.33</u>	<u>(G)<sub>8</sub></u>	<u>43–55</u>	<u>13–50</u>
<u>BLM</u>	<u>15q26.1</u>	<u>(A)<sub>9</sub></u>	<u>18</u>	<u>16</u>
<u>Caspase-5</u>	<u>11q22.3</u>	<u>(A)<sub>10</sub></u>	<u>46</u>	<u>62</u>
<u>CDX2</u>	<u>13q12.2</u>	<u>(G)<sub>7</sub></u>	<u>5</u>	<u>=</u>
<u>CHK1</u>	<u>11q24.2</u>	<u>(A)<sub>9</sub></u>	<u>100</u>	<u>10</u>
<u>DD5 (UBR5)</u>	<u>8q22.3</u>	<u>(A)<sub>8</sub></u>	<u>23</u>	<u>=</u>
<u>E2F4</u>	<u>16q22.1</u>	<u>(CAG)<sub>13</sub></u>	<u>71</u>	<u>42–57</u>
<u>ELAVL3</u>	<u>19p13.2</u>	<u>(G)<sub>9</sub></u>	<u>37</u>	<u>=</u>
<u>FLJ11383 (PCNXL2)</u>	<u>1q42.2</u>	<u>(A)<sub>10</sub></u>	<u>74</u>	<u>74</u>
<u>FLT3LG</u>	<u>19q13.33</u>	<u>(C)<sub>9</sub></u>	<u>38</u>	<u>35</u>
<u>hRAD50</u>	<u>5q31.1</u>	<u>(A)<sub>9/8</sub></u>	<u>45</u>	<u>31</u>
<u>HT001 (ASTE1)</u>	<u>3q22.1</u>	<u>(A)<sub>11</sub></u>	<u>85</u>	<u>=</u>
<u>IGF2R</u>	<u>6q25.3</u>	<u>(G)<sub>8</sub></u>	<u>13</u>	<u>9</u>
<u>MACS (MARCKS)</u>	<u>6q22.1</u>	<u>(A)<sub>11</sub></u>	<u>72</u>	<u>72</u>
<u>MBD4 (MED1)</u>	<u>3q21.3</u>	<u>(A)<sub>10</sub></u>	<u>28</u>	<u>25–40</u>
<u>MSH3</u>	<u>5q14.1</u>	<u>(A)<sub>8</sub></u>	<u>40–58</u>	<u>39–46</u>
<u>MSH6</u>	<u>2p16.3</u>	<u>(C)<sub>8</sub></u>	<u>24–33</u>	<u>28–36</u>
<u>NDUFC2</u>	<u>11q14.1</u>	<u>(T)<sub>9</sub></u>	<u>42</u>	<u>=</u>
<u>PTEN</u>	<u>10q23.31</u>	<u>(A)<sub>6</sub></u>	<u>18</u>	<u>13–34</u>
<u>PTHL3 (PTHLH)</u>	<u>12p11.22</u>	<u>(A)<sub>11</sub></u>	<u>86</u>	<u>90</u>
<u>RGS12</u>	<u>4p16.2</u>	<u>(C)<sub>8</sub></u>	<u>29</u>	<u>29</u>
<u>RIZ (PRDM2)</u>	<u>1p36.21</u>	<u>(A)<sub>8/9</sub></u>	<u>38</u>	<u>26</u>
<u>SEC63L</u>	<u>6q21</u>	<u>(A)<sub>10</sub></u>	<u>62</u>	<u>=</u>
<u>SLC23A1</u>	<u>5q31.2</u>	<u>(C)<sub>9</sub></u>	<u>40</u>	<u>45</u>
<u>SLC4A3</u>	<u>2q35</u>	<u>(C)<sub>9</sub></u>	<u>33</u>	<u>33</u>
<u>STK11 (LKB1)</u>	<u>19p13.3</u>	<u>(C)<sub>6</sub></u>	<u>=</u>	<u>2</u>
<u>TAF1B</u>	<u>2p25.1</u>	<u>(A)<sub>11</sub></u>	<u>78</u>	<u>82</u>
<u>TBP</u>	<u>6q27</u>	<u>(CAG)<sub>19</sub></u>	<u>=</u>	<u>83</u>

Formatted: Font: Not Bold

<a href="#">TCF1 (HNF1A)</a>	<a href="#">12q24.31</a>	<a href="#">(C)<sub>8/9</sub></a>	<a href="#">17</a>	<a href="#">23</a>
<a href="#">TCF4</a>	<a href="#">10q25.3</a>	<a href="#">(A)<sub>9</sub></a>	<a href="#">41</a>	<a href="#">39</a>
<a href="#">TEAD2 (TEF4)</a>	<a href="#">19q13.33</a>	<a href="#">(C)<sub>8</sub></a>	<a href="#">32</a>	<a href="#">32</a>
<a href="#">TFE3</a>	<a href="#">Xp11.23</a>	<a href="#">(G)<sub>8</sub></a>	<a href="#">24</a>	<a href="#">24</a>
<a href="#">TGFβRII</a>	<a href="#">3p22</a>	<a href="#">(A)<sub>10</sub></a>	<a href="#">77–83</a>	<a href="#">82–90</a>

[MMR, mismatch repair; MSI-H, microsatellite instability at high frequency; –, data not available.](#)

[\\*According to published mutation frequencies by <sup>56-58</sup>.](#)

[†The chromosomal locations are based on Ensembl release 50.](#)

[‡There is a statistically significant excess of APC frameshift mutations in MSI-H vs. MSS tumours](#)

[\( \$P < 0.001\$ \), despite the prevalence of APC mutations being the same in both tumour types <sup>59</sup>.](#)

[Note: the percentage refers to the respective number of the tumours studied in each case. For a full list of each individual study the reader is referred to the study by <sup>57</sup>.](#)

**Formatted:** Not Superscript/  
Subscript

**Formatted:** Not Superscript/  
Subscript

**Formatted:** Font: Italic

**Formatted:** Line spacing: Double



<b>Page 19: [1] Deleted</b> positive	<b>Bernard</b>	<b>7/8/2009 1:09:00 PM</b>
<b>Page 19: [1] Deleted</b> staining	<b>Bernard</b>	<b>7/8/2009 1:09:00 PM</b>
<b>Page 19: [2] Deleted</b> negative	<b>Bernard</b>	<b>7/8/2009 1:10:00 PM</b>
<b>Page 19: [2] Deleted</b> staining	<b>Bernard</b>	<b>7/8/2009 1:10:00 PM</b>
<b>Page 19: [3] Deleted</b> negative	<b>Bernard</b>	<b>7/8/2009 1:10:00 PM</b>
<b>Page 19: [3] Deleted</b> staining	<b>Bernard</b>	<b>7/8/2009 1:10:00 PM</b>
<b>Page 19: [4] Deleted</b> staining	<b>Bernard</b>	<b>7/8/2009 1:10:00 PM</b>
<b>Page 19: [4] Deleted</b> staining	<b>Bernard</b>	<b>7/8/2009 1:10:00 PM</b>
<b>Page 19: [5] Formatted</b> Font: Bold	<b>Bernard</b>	<b>7/8/2009 1:11:00 PM</b>
<b>Page 19: [5] Formatted</b> Font: Bold	<b>Bernard</b>	<b>7/8/2009 1:11:00 PM</b>
<b>Page 19: [6] Deleted</b> (	<b>Bernard</b>	<b>7/8/2009 1:11:00 PM</b>
<b>Page 19: [6] Deleted</b>	<b>Bernard</b>	<b>7/8/2009 1:11:00 PM</b>
<b>Page 19: [6] Deleted</b> )	<b>Bernard</b>	<b>7/8/2009 1:11:00 PM</b>
<b>Page 19: [7] Deleted</b> medium	<b>Bernard</b>	<b>7/8/2009 1:11:00 PM</b>

<b>Page 19: [7] Deleted</b> (x200 magnification)	<b>Bernard</b>	<b>7/8/2009 1:12:00 PM</b>
<b>Page 19: [7] Deleted</b> IHC	<b>Bernard</b>	<b>7/8/2009 1:12:00 PM</b>
<b>Page 19: [7] Deleted</b> positive	<b>Bernard</b>	<b>7/8/2009 1:12:00 PM</b>
<b>Page 19: [7] Deleted</b> staining	<b>Bernard</b>	<b>7/8/2009 1:12:00 PM</b>
<b>Page 19: [8] Deleted</b> positive	<b>Bernard</b>	<b>7/8/2009 1:12:00 PM</b>
<b>Page 19: [8] Deleted</b> staining	<b>Bernard</b>	<b>7/8/2009 1:13:00 PM</b>
<b>Page 19: [9] Deleted</b> negative	<b>Bernard</b>	<b>7/8/2009 1:13:00 PM</b>
<b>Page 19: [9] Deleted</b> staining	<b>Bernard</b>	<b>7/8/2009 1:13:00 PM</b>
<b>Page 19: [10] Formatted</b> Font: Bold	<b>Bernard</b>	<b>7/8/2009 1:13:00 PM</b>
<b>Page 19: [10] Formatted</b> Font: Bold	<b>Bernard</b>	<b>7/8/2009 1:13:00 PM</b>
<b>Page 19: [11] Deleted</b> Jan 15	<b>Bernard</b>	<b>7/8/2009 1:14:00 PM</b>
<b>Page 19: [11] Deleted</b> (1);	<b>Bernard</b>	<b>7/8/2009 1:14:00 PM</b>
<b>Page 19: [12] Deleted</b> Jan 7	<b>Bernard</b>	<b>7/8/2009 1:17:00 PM</b>
<b>Page 19: [12] Deleted</b> (1);	<b>Bernard</b>	<b>7/8/2009 1:17:00 PM</b>

Page 19: [13] Deleted	Bernard	7/8/2009 1:18:00 PM
-----------------------	---------	---------------------

*et*

Page 19: [13] Deleted	Bernard	7/8/2009 1:18:00 PM
-----------------------	---------	---------------------

Nov 16

Page 19: [13] Deleted	Bernard	7/8/2009 1:18:00 PM
-----------------------	---------	---------------------

;

Page 19: [13] Deleted	Bernard	7/8/2009 1:14:00 PM
-----------------------	---------	---------------------

-

Page 19: [14] Deleted	Bernard	7/8/2009 1:18:00 PM
-----------------------	---------	---------------------

*et*

Page 19: [14] Deleted	Bernard	7/8/2009 1:18:00 PM
-----------------------	---------	---------------------

Oct 13

Page 19: [14] Deleted	Bernard	7/8/2009 1:18:00 PM
-----------------------	---------	---------------------

;

Page 19: [14] Deleted	Bernard	7/8/2009 1:14:00 PM
-----------------------	---------	---------------------

-

Page 19: [15] Deleted	Bernard	7/8/2009 1:18:00 PM
-----------------------	---------	---------------------

Apr 10

Page 19: [15] Deleted	Bernard	7/8/2009 1:18:00 PM
-----------------------	---------	---------------------

;

Page 19: [15] Deleted	Bernard	7/8/2009 1:14:00 PM
-----------------------	---------	---------------------

-

Page 19: [16] Deleted	Bernard	7/8/2009 1:18:00 PM
-----------------------	---------	---------------------

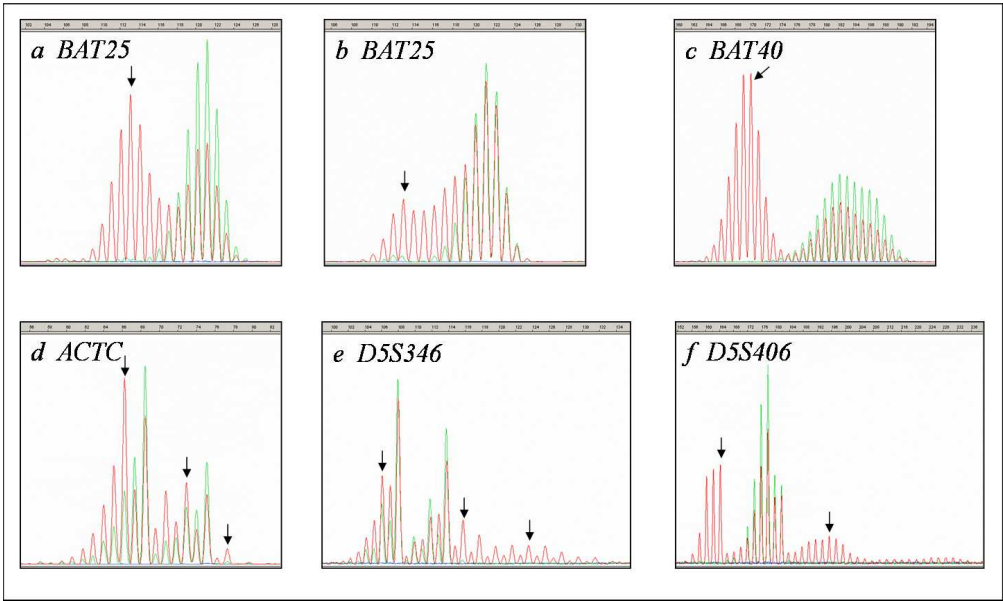
Dec 17

Page 19: [16] Deleted	Bernard	7/8/2009 1:18:00 PM
-----------------------	---------	---------------------

;

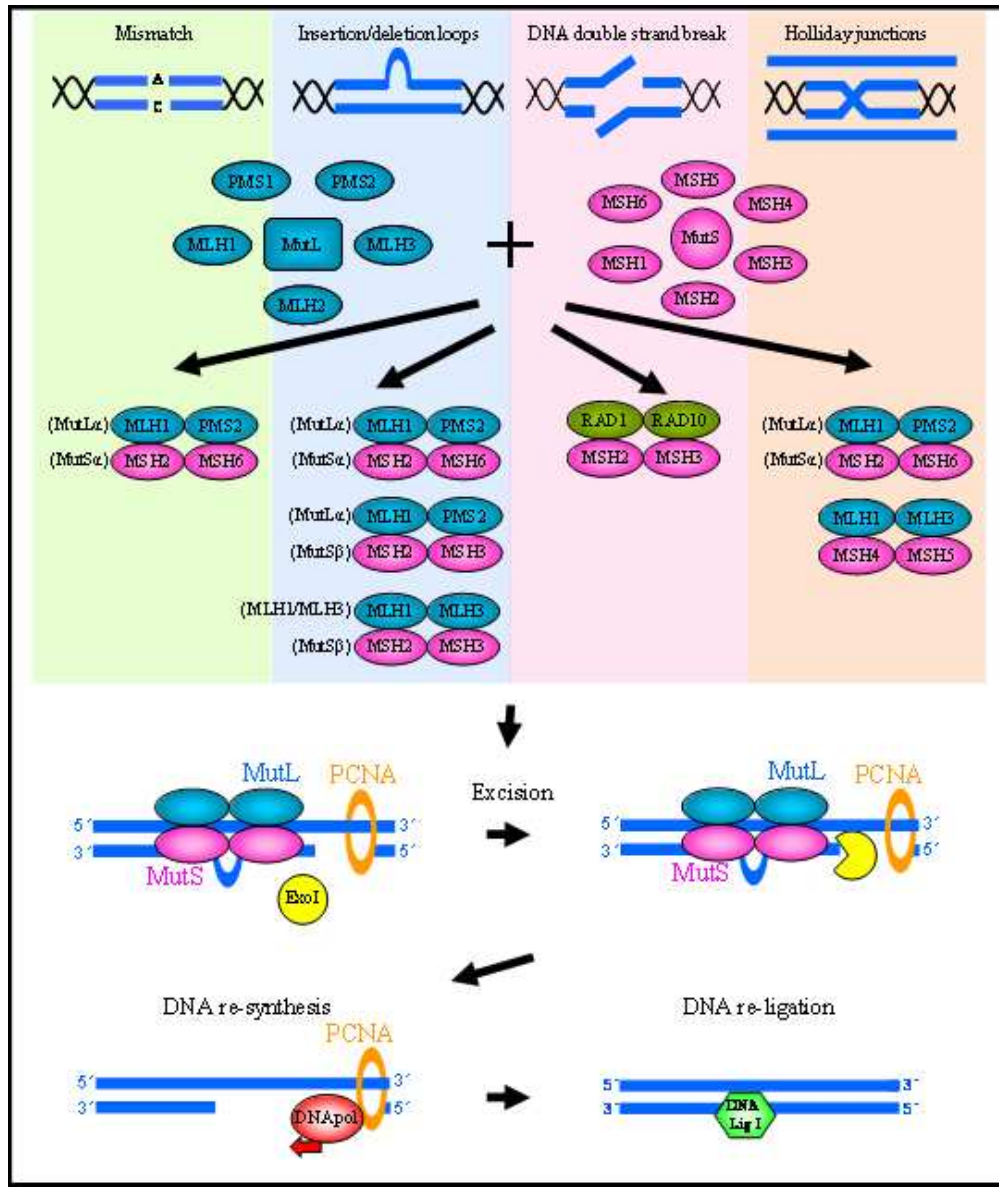
Page 19: [16] Deleted	Bernard	7/8/2009 1:14:00 PM
-----------------------	---------	---------------------

For Peer Review

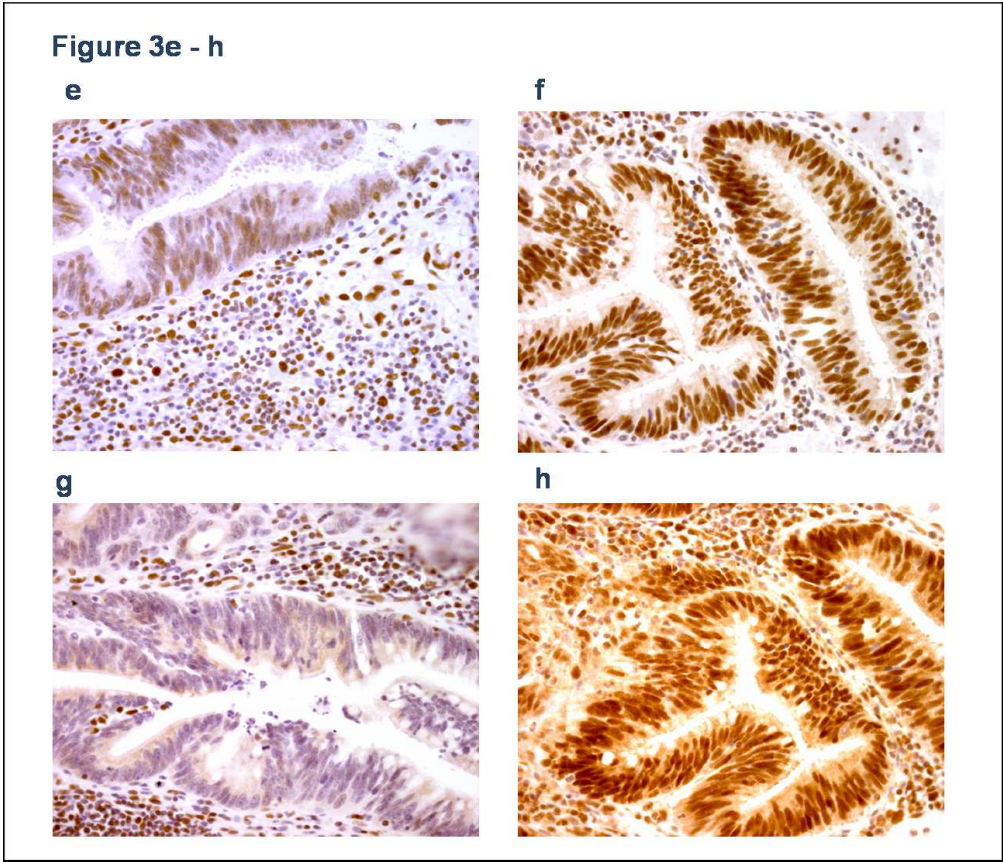


244x146mm (150 x 150 DPI)

er Review

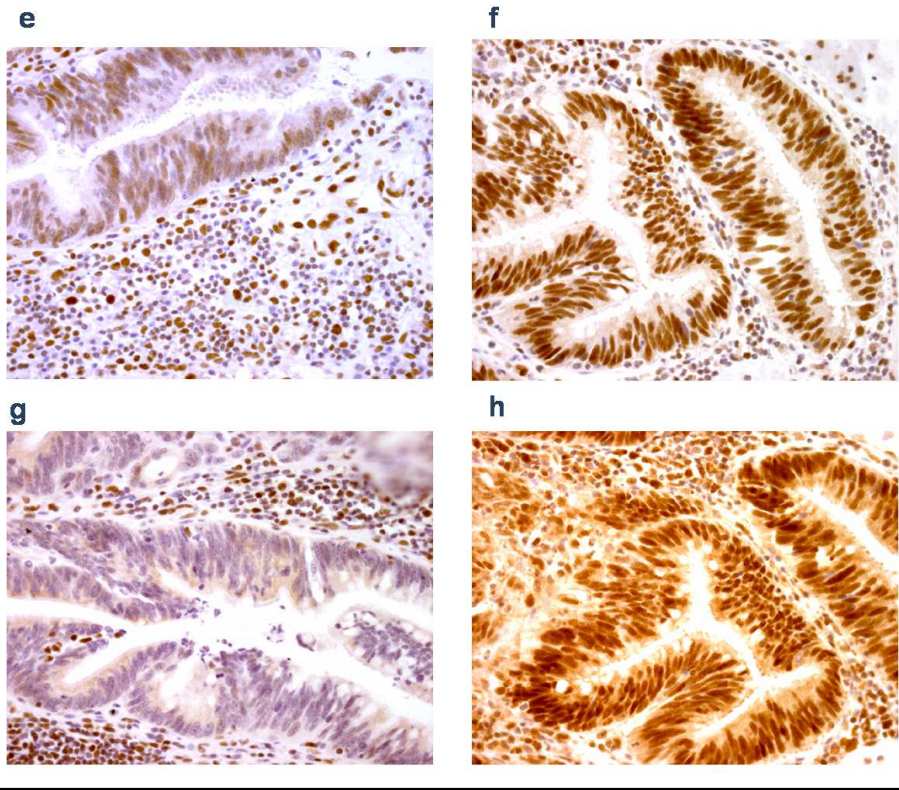


91x108mm (150 x 150 DPI)



218x187mm (150 x 150 DPI)

Figure 3e - h



218x187mm (150 x 150 DPI)