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SURVEY AND SUMMARY

Molecular models for the tissue specificity of DNA mismatch repair-deficient carcinogenesis

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ABSTRACT

A common feature of all the known cancer genetic syndromes is that they predispose only to selective types of malignancy. However, many of the genes mutated in these syndromes are ubiquitously expressed, and influence seemingly universal processes such as DNA repair or cell cycle control. The tissue specificity of cancers that arise from malfunction of these apparently universal traits remains a key puzzle in cancer genetics. Mutations in DNA mismatch repair (MMR) genes cause the most common known cancer genetic syndrome, hereditary non-polyposis colorectal cancer, and the fundamental biology of MMR is one of the most intensively studied processes in laboratories all around the world. This review uses MMR as a model system to understand mechanisms that may explain the selective development of tumors in particular cell types despite the universal nature of this process. We evaluate recent data giving insights into the specific tumor types that are attributable to defective MMR in humans and mice under different modes of inheritance, and propose models that may explain the spectrum of cancer types observed.

INTRODUCTION

DNA mismatch repair (MMR) and mechanisms of cancer prevention

More than 1 billion years old, MMR plays critical roles in the maintenance of genomic stability in prokaryotes, simple eukaryotes and metazoan organisms such as humans and rodents (1–3). Interest in DNA mismatch repair and its mechanisms of action exploded in the early 1990s with the observation that germline mutations in MMR genes cause hereditary non-polyposis colorectal cancer (HNPCC) (4–7). The biology of

MMR has been extensively characterized in model organisms, and these studies have given tremendous insights into the function of specific proteins in humans. The model organisms in which MMR has been most extensively studied are *Escherichia coli* and *Saccharomyces cerevisiae*. Other systems in which MMR genes have been rigorously characterized include *Caenorhabditis elegans* and *Arabidopsis Thaliana*. The precise mechanistic function that each MMR protein performs in these model organisms, as well as humans and mice, has been covered in detail in numerous excellent reviews (3,8–15). Therefore, this subject will be summarized only succinctly here, focusing on mechanisms most likely to be relevant to the discussion of tissue specific carcinogenesis.

Briefly, there are nine mammalian MMR genes (*MLH1*, *MLH3*, *PMS1-2* and *MSH2-6*) (3,8). The MMR proteins interact with each other to create a combinatorial code of complexes that mediate distinct functions. The mammalian *E.coli* MutS homologs (MSH proteins) are thought to directly contact double-stranded DNA, scanning along the genomic DNA for mismatches analogous to a 'sliding clamp' until they encounter a base pair containing a mismatch (16,17). The MSH proteins interact with multiple proteins including the mammalian *E.coli* MutL homologs (MLH) and yeast post-meiotic segregation (PMS) homolog proteins (which have significant amino acid identity and structural similarity to the MLH proteins), as well as RPA, EXO1, RFC, possibly HMGB1, and other less well-characterized proteins [reviews (3,8) as well as the new primary research papers (18–20) discuss this topic in excellent detail]. With respect to the mutator function, the MSH2–MSH6 heterodimer is thought primarily to repair single-base substitutions and 1 bp insertion–deletion mutations, while MSH2–MSH3 is thought primarily to repair 1–4 bp insertion–deletion mutations (3,8). The *E.coli* MutL homologs (MLH) and yeast PMS proteins interact with heterodimers of MSH proteins to help catalyze their different functions. MLH1–PMS2 is the primary MutL complex that interacts with both MSH2/6 and MSH3 complexes in mechanisms thought to be relevant to cancer

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prevention. Recent studies, including those from our laboratory, suggest mammalian MLH1–MLH3 also contributes to some of these processes as well, but in all mechanisms tested to a lesser degree than MLH1–PMS2 (21–24). MLH1–PMS1 clearly exists in mammalian cells but it currently has no clearly defined role in processes relevant to cancer susceptibility (25,26). *MSH4* and *MSH5* are thought to play roles exclusively in meiosis and are currently not thought to contribute to mechanisms of cancer prevention (27).

A mutator phenotype causing increased mutation rates for single-base substitution and insertion/deletion mismatches have traditionally been thought to be the main functions in MMR cancer prevention. Defects in the insertion/deletion repair function create a phenotype that is largely thought to be unique to MMR. Since this function was originally characterized on short repetitive DNA microsatellite sequences, it is often referred to as Microsatellite Instability or MSI (28–30). MSI is divided into MSI-Low and MSI-High (MSI-H) subtypes depending on the percentage of microsatellite markers that are mutated, but only the MSI-High subtype is clearly associated with defective MMR. Tumors with no MSI are referred to as Microsatellite Stable (MSS). [Consensus thinking on the topic of MSI was recently covered in detail by Umar *et al.* (31)]. The precise mechanisms whereby MSI causes cancer are hypothesized to include a genome wide increased mutation rate that causes mutations not only in microsatellites, but also in exonic coding sequences of genes important in cancer suppression. MSI mutation rates in these short repetitive sequences in *MLH1*- or *MSH2*-deficient cells are estimated to be more than 100 times higher than that seen in MMR-proficient cells and used to test directly whether MMR deficiency is involved in specific tumor types. Almost all described *MLH1/MSH2* mutations cause MSI-H tumors, although recently MSS *MLH1* non-synonymous amino acid substitutions causing deleterious mutations have been described (32,33). Current thinking is that MMR mutations are recessive (i.e. require two hits under the Knudson hypothesis) before cells become susceptible to cancer. However, it has been suggested that cells from subjects carrying heterozygous germline mutations may have detectable MSI using a more sensitive assay referred to as single-molecule or small pool PCR (34,35) that can detect approximately 5- to 10-fold elevations in MSI rates. These new findings are intriguing, but require further investigation before the role of MMR haploinsufficiency in cancer susceptibility is clearly established.

In addition to its essential roles in insertion/deletion and single-base substitution repair, MMR proteins also participate in additional mechanisms that could contribute to carcinogenesis, most notably initiation of apoptosis in response to DNA damage (16,36). Recent studies using mouse models of single-amino acid substitutions causing separation of function mutations have demonstrated clearly that decreased apoptosis plays an important role in MMR-deficient tumorigenesis (37,38). The precise mechanism by which MMR contributes to the initiation of programmed cell death remains unclear, with both futile cycles of repair causing high levels of double-strand breaks and direct signaling proposed (39,40). MMR mutant cells fail to activate a p73-dependent cell death activation pathway (41), which may explain the absence of a critical requirement for p53-mediated apoptosis

(42). This failure impairs cell cycle and DNA damage checkpoint recognition (3).

In summary, the current models suggest that MMR mutations cause cancer primarily through the contribution of both mechanisms: (i) cells acquire mutations in components of critical tumor suppressor gene pathways that allow them to proliferate and (ii) cells do not initiate apoptosis appropriately. It is unclear at present whether these mechanisms operate sequentially or concurrently in different cell types. MMR proteins also contribute to suppress homeologous recombination (39,43), which could in principle contribute as an important mechanism of carcinogenesis. However, the contribution of increased rates of homeologous recombination from MMR mutations to carcinogenesis is not well defined at this point in time.

The spectrum of cancer susceptibility attributable to MMR deficiency

Mutations in the MMR genes *MLH1* and *MSH2* were first identified because of their association with colorectal cancer in HNPCC families (4–7). Approximately 70–85% of HNPCC families have identifiable mutations in these two genes (9). Subsequently, HNPCC families with reduced penetrance and identifiable *MSH6* and *PMS2* mutations were identified (44,45). Further studies demonstrated that somatically acquired defects in MMR mutations are attributable for ~17 000 sporadic colorectal cancer (CRC) (46,47) and 10 000 endometrial cancer (48) new diagnoses in the United States each year. Germline *MSH6* mutations were also shown to be responsible for an appreciable proportion of familial colorectal cancer and hereditary uterine cancer families (49–51). More recently, pediatric patients harboring homozygous recessive *MLH1* or *MSH2* germline mutations have also been identified. In these individuals, the most notable susceptibility is to hematopoietic malignancies (which are not generally present in HNPCC) and colorectal cancer (52–56).

Numerous studies have attempted to characterize the tumor spectrum and age of onset of HNPCC-related tumors in affected individuals, but these studies have been fraught with challenges given the relative rarity of the syndrome and difficulty in gathering prospective data. Most studies have focused on identifying high-risk families of those that meet clinical criteria for HNPCC and comparing incidence data with that of the general population. However, this approach leads to biases in the data toward higher than actual rates of tumor development since the most strongly affected families tend to be included while those with a less severe phenotype are less frequently recognized (referral bias). HNPCC-Lynch Syndrome was originally described as a familial syndrome manifested by elevated incidence of colorectal and endometrial cancer and further studies have related this to MMR defects as well to numerous other cancer susceptibilities (4–7). Colorectal cancer has been reported in men with HNPCC with a cumulative lifetime risk upwards of 74% (57–59), and standardized incidence ratio of 9.6 (95% CI 7.5–12.3) in a clinic-based study of individuals with MSI-H tumors and as low as 2.0 (1.3–2.7) in the same study for population-based data in MSS individuals (60). In women, the risk of CRC is lower and lifetime risk of endometrial cancer has been

Table 1. Standardized incidence ratios (SIRs) of cancer risk by site in population-based and clinic-based ascertainment of MMR defective cancers

Tumor site	All MSI-H SIR (60)	Population-based SIR (60)	Clinic-based SIR (60)	Incidence per 100 000 (60)	Clinic-based SIR (63)	Incidence per 100 000 (63)	Combined incidence per 100 000 (60,63)
Colon	6.1 (5.2–7.2)	4.3 (3.4–5.3)	9.6 (7.5–12.3)	5319	N/A ^a		
Gastric	4.6 (2.7–6.6)	3.3 (1.4–5.4)	7.1 (3.1–11.7)	614	4	1291	802
Uterus	4.1 (2.9–5.6)	3.4 (1.9–4.8)	5.4 (3.1–7.9)	2440	N/A ^a	8049	4020
Kidney	2.6 (1.4–4.0)	1.8 (0.5–3.3)	4.1 (1.5–7.1)	438	3	759	528
Lung	0.3 (0.2–0.5)	0.2 (0.0–0.4)	0.6 (0.2–1.1)	351			
Ovary	2.0 (1.0–3.2)	1.6 (0.5–3.1)	2.6 (0.8–5.1)	714	3.5	1974 ^b	1069 ^b
Breast	0.5 (0.3–0.7)	0.4 (0.2–0.6)	0.6 (0.3–1.0)	1250	<1		
Hematopoietic	0.5 (0.2–0.8)	0.7 (0.2–1.1)	0.3 (0.0–0.7)	321	1		
Pancreas	1.7 (0.7–2.8)	2.1 (0.8–3.8)	1.0 (0.0–2.7)	292	1		
Prostate	0.3 (0.1–0.5)	0.3 (0.1–0.6)	0.3 (0.0–0.7)	292			
Small bowel	7.6 (2.5–13.9)	9.7 (1.9–19.3)	3.6 (0.0–12.0)	175	25	759	338
Cervix	0.3 (0.1–0.5)	0.2 (0.0–0.4)	0.5 (0.0–0.9)	357	1	1063 ^b	556 ^b
Bladder	0.4 (0.1–0.9)	0.3 (0.0–0.7)	0.8 (0.0–1.8)	146	1	380	211
Hepatobiliary	2.4 (0.6–5.0)	0.9 (0.0–3.2)	5.3 (0.0–11.7)	117	5	532	232
Ureter	9.0 (2.0–18.3)	10.0 (0.0–22.9)	6.8 (0.0–21.8)	117	22	380	190
Brain	0.7 (0.0–1.7)	0.7 (0.0–1.9)	0.6 (0.0–2.1)	88	1		
Head/neck	0.3 (0.0–0.6)	0.4 (0.0–0.9)	N/A	88	1		
Melanoma	0.3 (0.0–0.4)	0.2 (0.0–0.6)	N/A	58			

^aIn this study, these cancers were used to ascertain high-risk families, therefore an unbiased estimate of incidence cannot be estimated.

^bEstimated 50% of patients were female in this study.

Relative risk is presented with 95% confidence intervals in parentheses. Data are compiled from (60,63).

reported as low as 30% (61) in a study based on registry data from Finland, Holland and the United States. Other studies have placed the lifetime risks of endometrial cancer as much higher: 42% for women with a mutation in *MLH1*, 61% for *MSH2* (58). While the elevated risk for both CRC and endometrial cancer in these patients is undisputed, it soon became clear upon observing these patients and their families that cancers at other specific sites seemed to appear far more frequently than one would see in the general population. Most reliable studies have relied on the calculation of standard incidence rates to evaluate these observations, i.e. the ratio of observed to expected cancer rates compared with population based information. The first study on this subject demonstrated an elevated SIR for gastric cancer (SIR = 4.1), hepatobiliary (SIR = 4.9), urologic tract (SIR = 2.5), ovarian (SIR = 3.6), and small bowel cancer (SIR = 25), as well and an increased but not statistically significant SIR of 1.6 for brain tumors (62) which has not been substantiated by further studies. Further work in this area has verified these results, and has repeatedly shown increased risk of small bowel, hepatobiliary, gastric, skin keratoacanthoma, ureter/kidney and ovarian tumors. Anecdotal reports of tumors such as leukemia, lymphoma, pancreas, larynx and breast tumors have not been substantiated by larger scale observational studies (60,63).

Recent large-scale population and clinic-based analyses have been performed that have less overall bias than single institution case series (60,64–66). These studies use MSI or loss of immunohistochemical staining as the screening methodology, and therefore identify both germline and sporadic MMR defects. In these studies, the greatest cancer susceptibility is manifest as colorectal cancer [odds ratio (OR) 4.3–9.6], endometrial cancer (OR 3.4–5.4), stomach cancer (OR 3.3–7.1), kidney (OR 1.8–4.1), ovary (OR 1.6–2.6), small intestine (OR 3.6–9.7) and ureter (OR 6.8–10.0) (Table 1) (60). Surprisingly, significantly lower cancer rates are also observed, most convincingly and repeatedly seen in prostate, breast and

lung cancers (60,63,67). Environmental factors have also been demonstrated to play a role in the affecting the tumor spectrum. Recent data from HNPCC mutation carriers support the assertion that smoking significantly increases CRC risk in HNPCC mutation carriers (hazard ratio of 1.43, $P < 0.04$) (68).

The spectrum of cancer susceptibility in germline homozygous MMR mutation mouse models

Mouse models for all nine MMR genes, as well as numerous combinations of these genes, have been created and have given significant insights into the mechanisms and cancer susceptibility of specific cell types (25,39,69–77). *Msh2*^{-/-} and *Mlh1*^{-/-} mice have a highly penetrant cancer susceptibility, which manifests primary as lymphomas, GI epithelial adenomas and basal cell carcinomas starting at ~6 months of age (25,39,76,78). *Msh6*^{-/-} mice display essentially the same tumor spectrum, but with the same tumor types appearing around 9–12 months of age (72). *Pms2*^{-/-} mice develop lymphomas and sarcomas but not GI tumors, starting around 9–12 months of age (25,79,80). *Mlh3*^{-/-} mice have lower penetrance GI cancer susceptibility and develop GI cancers with a mean onset of 12 months of age, as well as lymphomas and skin cancers (23). *Pms1*^{-/-}, *Msh3*^{-/-}, *Msh4*^{-/-} and *Msh5*^{-/-} mice have to date demonstrated no clear susceptibility to cancer (25,71,81). In summary, the tissue specificity of all MMR mouse knock out models is very similar, but with varying penetrance. In mice, the main tissues affected are GI epithelial tumors and mixed B and T cell lineage lymphomas. It is not well understood at present why endometrial, ovarian and renal cancers do not occur with appreciable frequency as they do in humans. It has been speculated that this difference might reflect the shorter lifespan of mice, the lower penetrance of these other cancer types, differences in hormonal exposure of the estrous versus menstrual cycles or environmental exposures.

Contribution of the mutator phenotype: distinct sequences have higher mutation rates in genes critical for different cell types

Since different cell types have distinct critical tumor suppressor and tumor promoter gene requirements, the precise sequences of these genes in part may help define the tissue specificity of MMR defects as mutation targets. It is well appreciated that different cell types have distinct patterns of gene expression/splicing (82,83) and are dependent on distinct tumor suppressor and tumor promoter genes [for recent review, see (84)]. Therefore, it is not surprising to accept the premise that specific cell types are more susceptible to perturbations in specific signaling pathways than others. It is clear that defective MMR preferentially causes specific sequences to mutate at faster rates than other sequences. The best-characterized highly specific preference is for mutation of short pair repetitive sequences, a phenomenon originally described by Perucho, Shibata, Thibodeau and colleagues (28,29). Current thinking is that insertion/deletion mutations of most non-coding genomic microsatellites do not contribute directly to cancer susceptibility, but that these mutations do play a role when they occur in coding or proximal promoter regulatory sequences. Perhaps the best-characterized target is the transforming growth factor β type II receptor, which in humans has a 10 Adenine repeat in its coding region that is frequently mutated in MSI-H CRC tumors (85). Since TGF Beta signaling is particularly significant for GI epithelial cells, and repression of this pathway's signaling is relieved by transforming growth factor beta type II receptor (*TGFBR2*) adenine repeat frameshift mutations that occur at high rates in MMR defective cells, the combination of these two events may explain in part why GI epithelial cells are preferentially transformed in HNPCC. However, because this 10 adenine coding repeat is not present in mouse *Tgfr2* and the MMR knockout models develop GI carcinomas, it cannot be the only explanation for the preference of tumors to develop from this cell type. Recent genome wide surveys have cataloged genes whose coding sequence contains repetitive mono- and dinucleotide repeat sequences (86–88) that are likely candidates for frameshift mutations in MMR-deficient cells. Because nonsense-mediated decay (NMD) causes the degradation of many mRNAs with frameshift mutations causing premature stop codons, cross comparisons of different gene expression patterns in MSI-H cancers versus normal mucosa with the list of genes encoding longer repetitive mono- and dinucleotide repeat sequences are a well focused strategy to identify selective targets in different tissues that explain the predilection of MMR defects to cause specific cancer types. Experiments in cell lines using this approach and selective stabilization of NMD targeted mRNA transcripts with chemical or genetic approaches has suggested *UVRAG*, *p300* and *GRK* as potential important targets in MSI-H CRCs (89,90). It will be interesting to see how the targets identified by this approach when tested in other cell types such as uterine epithelial cells, or use primary cancer cells.

There is also evidence that even mildly repetitive sequences in genes whose function is critical are selected with higher frequency by MMR deficiency as common mutation targets. For example, the spectrum of *Apc* mutations in MMR-deficient tumors has been exhaustively characterized in mouse models.

In mouse GI epithelial tumors, *Apc* deletions of a single 'CA' in TCC CAC ACA CA or a single 'GA' in AAG AGA GAG AG, both of which are in the *Apc* coding region, cause frameshift truncation mutations have each been observed more than 20 times and occur significantly more frequently than any base-substitution mutation (91–93). Similar observations have been made in human MSI-H colorectal tumors (94). Therefore, the contribution of the MMR mutator function to cancer cell type specificity is likely to involve elements of (i) the presence of repetitive coding sequences in specific genes, (ii) the expression pattern of those genes and (iii) the functional importance of those genes so that even rare mutations can be selected for if they confer a proliferative advantage.

How do targets of the MMR mutator phenotype influence tumor specificity?

The observation that human and mouse null mutations for critical MMR genes allow essentially normal development demonstrate clearly that the loss of MMR alone is insufficient to initiate a carcinogenic transforming event, but that additional genetic changes are also required for the transition to frank malignancy. The genes that are inactivated by frameshift mutations in MMR-deficient cancers have been evaluated by several groups (95,96). Of all the genes, *TGFBR2*, *BAX* and *CASP-5* stand out as the most consistently mutated in MMR defective tissues (95,96). *TGFBR2* is consistently mutated in most MSI-H cell lines and CRC tumors with 1 or 2 bp deletions in a coding poly(A)10 tract causing a frameshift mutation (85) and this finding has been consistently replicated by multiple groups (95–97). Subsequently, multiple other genes with putative tumor suppressor functions and containing coding microsatellites have shown to be mutated at various frequencies in sporadic MSI-H and HNPCC associated colorectal, gastric and endometrial cancers. These include additional genes such as *IGFIIR*, *PTEN* (98) involved in signal transduction, apoptosis (*BAX*, Caspase 5) (95,99), DNA repair, *MSH3*, *MSH6*, *MBD4* (100,101), as well as genes that play roles in transcriptional regulation, protein stability and immune surveillance. It is thought that by analyzing different cell lines, coding repetitive sequences that are not under growth selection (found in either tissue or temporally-specific non-transcriptionally active genes) or which are essential to cell survival would only appear with a low frequency proportional to that of the mutation rate of the MMR-deficient cells. These genes are sometimes referred to as 'bystander' or 'survivor' genes' (102). One would expect a bias toward decreased mutation frequencies in these coding MS (cMS). Finally, that genes in which frameshift mutations would convey a growth or selective advantage to the cell, presumably by abrogation of some tumor suppressor function, can be identified by a higher than expected frequency of mutation in MSI-H tumors. Duval *et al.* (103) used the maximum likelihood statistical analysis to search for new genes in which coding repetitive sequence mutations would provide a selective advantage to growth or tumor progression in a group of 9 known target genes and 19 others with an expected role in carcinogenesis. They also analyzed a series of 22 non-coding versus 25 coding short mononucleotide repeats (≤ 10 bp) where 7/25 coding repeats (in *CASP5*, *MSH6*, *IGFIIR*, *GRB14*, *BAX*, *TCF-4* and

TGFβ2R) showed a significantly higher mutation frequency; however, a great amount of variability was seen with mutation frequencies ranging from 2 to 92% in coding, and 0 to 40% in non-coding sequences (104). Other approaches have (102) used comprehensive literature search to identify 169 coding and 25 non-coding repeat sequences in colon, gastric and endometrial cancer and a cumulative mutation model to predict the genes with the strongest evidence for involvement in MMR defective mechanisms of carcinogenesis in the colon. These genes included *TGFβ2R*, *BAX*, *TCF-4*, *MSH3*, *ACVR2*, *PTHL3*, *HT001*, *AC1*, *SLC23A1*, *MACS*, *NDUFC2* and *TAF1B* (88,102). Two other genes, *WAF1* and *BCL2*, were found with mutation frequencies below the lower prediction limit arguing for negative selection in these cases. If this prediction is correct, it could be tested by creating models of MMR deficiency combined with *Waf1* or *Bcl2* null mice. It follows that observation of decreased tumor incidence or a shift in tumor spectrum would provide further evidence for a negative selection bias against these cMS. Similar techniques have also been used to discern a temporal relationship between target genes in the progression from adenoma to full blown malignancy emphasizing the likelihood that loss of *BAX*, *TGFβ2R*, *MACS*, *NDUFC2*, and *TAF1B* as early events and potential therapeutic targets (88).

Multiple studies have continued to identify a small subset of genes with coding repetitive sequences whose loss of function mutations likely contribute to tissue specificity of carcinogenesis. More problematic, has been the question of tissue specificity of these genes and pathways since there are obvious differences in target genes according to tissue type. This has been most frequently studied with regard to attempts to distinguish genes specific for either colorectum stomach or endometrium carcinomas. The spectrum of target genes in colon and gastric cancers appears very similar (97,105) with the exception of two genes, *TGFβ2R* ($P = 0.0004$) and *MSH6* ($P < 0.0001$). Overall, endometrial cancers showed far less instability with mutation frequencies of *TGFβ2R*, *MSH6* and *MSH3* were significantly lower than in gastrointestinal than endometrial tumors ($P < 0.01$). As predicted, in all three cancer types the distribution of mutation frequencies in putative target genes was bimodal suggesting a peak for genuine target genes and ‘bystanders’ at lower frequencies (102).

Why GI epithelial cells and where along the proximal-distal axis?

Because mutation rates are significantly higher in MMR defective versus proficient cells, current thinking is that the cell types with the highest overall proliferation rate (and therefore the total number of genomic replication cycles) would be most susceptible to cancer susceptibility attributable from the MMR mutator phenotype (3,101,106,107). This observation is likely to contribute to the spectrum of MMR cancers. In humans, the GI epithelium has the highest known proliferation rate of all cell types that have been measured and is thought to turn over approximately every 3–5 days (108). Other cell types that cycle rapidly include bone marrow, skin and epithelial cells of the upper GI tract. The *in vivo* ‘experiment’ of which cells have the most rapid proliferation rate is repeated daily in patients with cancer who are treated with standard cytotoxic

chemotherapy agents. These drugs target universal processes in proliferation such as genome replication by DNA polymerases or unwinding by topoisomerases and kill the cells with the highest proliferation rates. Acutely, patients develop diarrhea, which reflects cell death of lower GI epithelium. In the next few days later, red, white and platelet blood cells nadir, hair falls out, and mucositis develops reflecting the slightly less rapid turnover rates of bone marrow progenitor, hair/skin and upper GI epithelial cells. It is likely that these high proliferation rates explain in part the high rates of lower GI epithelial tumors that results from MMR mutations, although there is a paucity of direct experimental evidence to support the assertion. However, it is clear that ranking cell types by proliferation rates alone does not explain why other tissues, such as the genitourinary tract or ovarian epithelium, are affected. Therefore, additional causes must exist as well.

MMR-environment interactions in the gastrointestinal and genitourinary epithelium

MSH2–MSH6 can specifically recognize distinct types of damaged DNA (such as those caused by oxidative or alkylating agents). It has previously been proposed that the tissues susceptible to malignancy in the context of defective MMR could be explained by a specific type of DNA damage occurring primarily at these sites (16). The affected cells would not correctly initiate apoptosis to this damage, thereby leading to a selective advantage and transformation (16,109–111). While a specific type of DNA damage has not to date been shown to be unique to the cell types affected by MMR deficiency (and absent in the unaffected cell types), environmental insults have been demonstrated to play a role in the affecting the tumor spectrum in both patients and mouse models. Recent data from HNPCC mutation carriers support the assertion that smoking significantly increases CRC risk in HNPCC mutation carriers (hazard ratio of 1.43, $P < 0.04$) (68). Ethnic background also affects tumor spectrum (112–114), and marked inter-family variation in risk of HNPCC spectrum cancer is clearly documented (58,67,115–120). The variance is significant in the expressivity of the syndrome (i.e. the distribution of different tumor types that develops in different HNPCC families) (115). However, the precise environmental (or other genetic) modifiers that influence these phenotypic variances are incompletely characterized.

It is notable that changes in relative tumor susceptibility of the stomach, small intestine and colorectum are observed to vary with both the temporal era as well as geography. The first HNPCC family identified was described by Aldred Warthin, a pathologist in 1913, in Europe and is referred to as Family G (121,122). Members of the original family are still alive today, and descendents have been followed in Europe and the United States. Similarly, many other HNPCC kindreds have family histories that extend over a century (48,122). In the early 20th century, the GI tumors in Family G and other similar kindreds were distinguished largely by an excess of stomach adenocarcinoma (123). In mid and late 20th and early 21st centuries, the GI cancer susceptibility has shifted to include mostly colorectal cancers. There are also notable differences between the cancer spectrum of HNPCC families that have been identified in Asia, Europe, Australia, North and South America and Africa. For example, it is of note

that the GI cancer susceptibility of Asian families generally has greater incidence of stomach cancer compared to North America, South America and Europe, where colorectal cancer predominates (118,119,122). It has been speculated that the shift in the GI cancer susceptibility rostral-caudal axis has reflected changes in food preparation and content. In particular, the decreased use of 'smoked' and cured meats resulting from refrigeration, and the high fat content of the modern European-American diet have been proposed as important factors (122). The smoking process of meats is thought to introduce heterocyclic amine byproducts in the food, and the curing process involved nitrate salts that cause nitroso-compounds, which are thought to act as potential mutagens. Epidemiologic and animal model studies provide evidence for this model (68,124). Direct experimental evidence supports the epidemiologic hypothesis that specific environmental mutagens play important roles. Cell culture experiments using *Msh2*^{+/-} cells in culture demonstrate that treatments with the genotoxic agents such as alkylators (ethylnitrosourea) or DNA cross-linkers (mitomycin C) selects for *Msh2*^{-/-} cells that increase in relation to the number of *Msh2*^{+/-} heterozygous cells (125). Part of this selection may reflect the observation that MMR cells appear to have higher overall proliferation rates compared to MMR-deficient cells, which would give them a growth advantage.

In MMR mouse models, the relative susceptibility of GI epithelium along the rostral-caudal axis (stomach, small bowel and colorectum) to form tumors is modulated by dietary fat and pharmaceuticals. Diets that are high in fat and low in calcium and folate (commonly referred to as 'Western Diets') increase most significantly the incidence of colorectal tumors compared with small intestine or stomach tumors (126), which parallels the higher colorectal cancer rates observed in HNPCC families in North America and Europe. Non-steroidal anti-inflammatory agents (NSAIDs) and PPAR gamma agonists can decrease small intestine tumors, while increasing stomach and colon tumors in MMR-deficient mouse models (127,128). Two randomized prospective human chemoprevention trials involving NSAIDs and HNPCC family members are ongoing, and more precise data as to how well the mouse model findings can be extended to humans will be available in the next several years (129,130). *CDX2* is a transcription factor that helps specify GI epithelial cell fate through a rostral-caudal expression gradient. Mutations in *Cdx2* are implicated with carcinogenesis in GI epithelium (131,132). As *CDX2* down-regulation is frequently observed in colorectal cancer and mutations and lower expression are strongly associated with MSI-H CRCs (133), it would be interesting to test directly, perhaps using mouse models, whether dietary modulation of *Cdx2* function might be an important mechanism that underlies the shifts along the different epithelia along the lower GI tract for MMR defective tumors.

While no precise environmental exposures or dietary or pharmaceutical agents have been specifically implicated in the non-GI MMR tumor spectrum sites, it is proposed that such agents concentrated in the urine may contribute to the higher rates of renal and ureteral cancers seen in HNPCC (134). Since cadmium is associated with both inactivation of MMR (135) and sporadic renal carcinoma (RR 2.0) (136,137), testing the hypothesis that this agent induces

MMR-deficient renal cancers could be carried out by exposing MMR defective animal models, or measurements of this element in renal tissues from affected patients as cadmium should not be affected by formalin fixation. Other potential agents to test prospectively in these models associated with renal cell cancer risk include asbestos, trichloroethylene and other petroleum by-products that are concentrated in urine.

Target genes in tumors from germline recessive versus sporadically acquired MMR deficiency

Patients with germline recessive MMR mutations (two mutant alleles) have a different spectrum of tumor types that that develops with dominant MMR (one mutant germline allele and somatic loss of the second allele, or sporadic functional loss of both alleles). Compared with HNPCC, recessive *MLH1*, *MSH2*, *MSH6* and *PMS2* mutations cause GI epithelial cancers as well as acute and chronic myeloid leukemias, Non-Hodgkin's lymphomas, dermal neurofibromas characteristic of Neurofibromatosis Type I and perhaps glioblastomas (52-56,138).

The observation that the cancer susceptibility of recessive versus dominant MMR mutations is different may give clues as to potential target genes. In particular, the leukemia susceptibility of patients with germline recessive MMR mutations suggests an interesting experimental model. This model would predict that there exists a primitive myeloid progenitor cell type during early development (i.e. present in individuals who inherit two MMR mutant alleles) not present in adults (such as in patients who inherit one mutant allele and have somatic loss of the second allele) that contains (i) target genes particularly sensitive to MMR-deficient mutation, or (ii) critical dependence on MMR mediated apoptosis. Furthermore, this model would also predict that this primitive myeloid progenitor cell type is rare or absent during adult life, and therefore not susceptible to transformation by dominant MMR mutations with subsequent loss of the second allele. In support of the first prediction by this model, mechanistic studies in mouse models looked at MMR germline defects combined with mice that are mutant for *Nfl*, the gene mutated in Neurofibromatosis Type I. *Mlh1*^{-/-}; *Nfl*^{+/-} mice develop myeloid leukemias, similar to the hematopoietic malignancies observed in patients with recessive MMR mutations (139). The tumors had significant MSI-H mutations, and loss of neurofibromin expression in all tumors analyzed. These results suggest that MMR deficiency can accelerate myeloid leukemogenesis in *Nfl*^{+/-} mice, presumably by inactivating *Nfl* gene expression as a myeloid lineage tumor suppressor gene. As there is substantial evidence that inherited versus sporadically acquired mutations significantly influence the types of tumors that develop [reviewed in (140)], and all the current described MMR mouse models use germline mutations, future experiments involving inducible *Mlh1* or *Msh2* mutations using Cre-Lox technology in ovary, kidney or uterus will be important to test whether these models develop cancers in those tissues.

Immune surveillance and tissue specificity of cancer susceptibility

The insertion/deletion frameshift mutation phenotype of MMR deficiency is predicted not only to cause gene mutations, but also lead to the creation of novel protein antigens

that are generated by the alternate reading frames and expressed in cells. It has been suggested that additional tumor types may develop in MMR null patients, but that they are suppressed by the immune system. In support of this concept, MSI-H CRCs are associated with a higher concentration of infiltrating lymphocytes than are MSS CRCs, and this pathological finding is included as one of the features to help physicians screen for MSI-H tumors (141).

Because MMR null mouse models develop NHL while HNPCC patients generally do not, a recent study screened a large series human NHLs for MSI. The MSI-H phenotype was present only in patients with HIV or iatrogenic (post bone marrow transplant) induced immunodeficiency (142). These data are consistent with a model whereby helper T-cell mediated immune surveillance does play a role in suppressing NHLs that develop in the context of MMR deficiency (presumably in these cases due to homozygous somatic inactivation), and thereby contributes to limiting the clinically apparent cancers in patients. The observation is supported by mouse model studies that show combining MMR deficiency with immunodeficiency (*Msh2*^{-/-};*Foxn1*^{-/-} and *Msh6*^{-/-};*Foxn1*^{-/-}) significantly increases NHL susceptibility (143). Why immunocompetent MMR homozygous mutant pediatric patients and mouse models develop NHLs is unclear, but the parallel findings suggest the same mutator or anti-apoptotic targets may be affected in both species. The identification of frameshift-inactivating mutations of pro-apoptotic factors *BAX* and *Caspase-5* in the NHLs from immunodeficient patients predicts they may be affected in the mouse models. However, since mouse *Bax* lacks the polyguanine repeat present in human *BAX* and the mouse ortholog of *Casp5* is controversial, these experiments would require 'humanizing' these genes in mice before analyzing mouse lymphomagenesis.

Tissue specificity of apoptosis mechanisms in MMR defective cancers

Failure of apoptosis is considered to be one of the six fundamental hallmarks of malignant disease (144) and clearly plays a major physiologic role in MMR-deficient tumors (145,146). These observations provide a preliminary basis for the examination of the role of apoptosis in MMR defective cell type specific cancer susceptibility. There are three reasons for thinking that initiation of apoptosis may contribute to MMR defective tumor specificity. First, current quantitative biological models of colorectal cancer emphasize abrogation of apoptosis as a very early stage in progression (147,148). These integrative biology models provide evidence that defects in apoptosis precede increases in mutation rates or genomic instability, which are thought to affect progression. Second, different cell types have distinct critical requirements for different genes in apoptotic signal transduction pathways. These cell type specific requirements have been characterized in different tissues, with the best-characterized example the somatic selection for *BCL2* expression in B-cell lymphomas (149). Other mutations in apoptotic genes, in contrast, are critical for other cell types. For example, *Caspase-9*, *Cytochrome C* and *Bax* cell models demonstrate critical roles for these genes in neural apoptosis, while *caspase-8* and *FADD* are critical in cardiomyocyte apoptosis (150). In wild-type and

MMR defective cells, there is preliminary evidence that apoptosis may be mediated through a PARP-1 dependent but caspase independent mechanism (93,151). Since there exist selective blockers of PARP-1 (DHIQ and DPQ) and caspase inhibitors (BAF and Z-VAD.fmk), studies to investigate the precise contribution of these different apoptotic pathways in different cell types that are either susceptible or resistant to MMR defective carcinogenesis may be informative whether critical signaling defects correlate with cell types susceptible to defective MMR-induced carcinogenesis. It is notable that *BAX*, which helps mediate Caspase-3 inducible apoptosis, is a common target of MMR mutations. It is underappreciated that mathematical and computer models of multistage theories of progression can be used to predict cancer rates in different cell types from quantitative aspects of apoptosis and DNA repair (152). If precise quantitative data on cell proliferation and mouse model tumor incidence data can be collected from tissues that are susceptible and resistant to MMR defective carcinogenesis (colorectal and breast epithelium for example), specific predictions of the relative contributions of apoptosis can be used to predict which additional tumor types would be susceptible to MMR inactivation by using age-specific penetrance, stage and metachronicity parameters. Third, apoptosis is particularly critical for all the cell types that are susceptible to cell autonomous MMR deficiency, including gastrointestinal, ovarian and endometrial epithelium and white blood cells. As discussed, many of the most crucial members of apoptotic pathways for these organ sites can be targeted MMR-deficient mutation, most notably *BAX*, *APAF-1*, *BCL10*, *CASP-5*, *FAS* and others (105).

In particular, the role that apoptosis plays in uterine endometrium and ovarian epithelium as part of normal tissue physiology is underappreciated. In response to hormonal stimulation, the uterine endometrium undergoes monthly cycling including a proliferative or follicular (pre-ovulatory), secretory or luteal (post-ovulatory) and menstrual phases. During each of these phases, there is marked change in the quantity of and quality of the endometrial cells. The proliferative phase is marked by high mitotic index, and Ki-67 indicative of rapid cellular proliferation, as well as elevated levels of *BCL2*, an anti-apoptotic factor, presumably involved in stabilizing the cells during this period and allowing for normal physiologic growth in preparation for implantation and pregnancy (153). During the luteal phase, *BCL2* expression falls, consistent with the appearance of apoptotic cells, and with higher expression of Fas, Fas-L and elevated activity of caspases 3, 8 and 9 from the luteal phase to menstruation (154). These cycle-related changes in expression are most prominent in the glandular epithelial cells where menstrual-related endometrial thickening and sloughing occurs, as well as the most common site of endometrial carcinogenesis in MMR deficiency. More consistent basal levels of *BCL2* expression are maintained in basal laminal, stromal and epithelial cells, where escape of apoptotic death is necessary for these cells to rebuild the functional endometrium with the next hormonal cycle. Similar studies in the ovary demonstrate the importance of programmed cell death in this organ. During the menstrual cycle, a single mature follicle is released by rupture of the ovarian epithelium. Multiple studies have implicated apoptosis and related signaling pathways as the mechanism of ovarian cell death (155,156). *BCL2* expression is markedly elevated during the

luteal phase and early pregnancy, but notably absent in the regressing corpus luteum (157). Assays of DNA fragmentation as a marker for apoptotic cell death showed high molecular weight DNA during early luteal phases, while mid-luteal phase DNA demonstrates cleavage into low molecular weight ladders (158).

The critical role of apoptosis in the susceptibility of ureter and urologic system, however, is less clear. Urothelial cells have a relatively low basal rate of proliferation and apoptosis in order to maintain tissue homeostasis (159); there is some evidence that this rate changes over time with exposure to toxins and other environmental exposures in the urine. This may also lead to potential apoptotic cell death and entry into wound healing pathways; however, the evidence for apoptotic signaling pathways contributing to GU cancers is less clear.

Acceleration and deceleration of proliferation: are the rates of change of proliferation rates important?

Although less intensively studied than the GI tract, it is clear that uterine endometrial and ovarian epithelial cancers are susceptible to defective MMR (Table 1). A study recently compared on a large-scale GI epithelial and uterine endometrial cancers as to which MSI susceptible genes contain frameshift mutations (97). This study suggested that the profile of target gene mutations demonstrates both qualitative and quantitative differences between gastrointestinal and endometrial MSI-H cancers. For colorectal and gastric MSI-H tumors, the genes affected were essentially identical. Mutations in the DNA-dependent protein kinase catalytic subunit, Glnac, CCAAT-box binding factor and *CtBP*-interacting protein coding regions appeared to be specific for endometrial but not GI MSI-H tumors (97). However, they do not clearly establish cell type specific GI-uterine targets that would not be expected to play roles in other tissues as well. In MMR-deficient mouse model experiments, *Pten*, a gene mutated also in human MMR defective cancers (103), was shown to be an important target whose mutation increases endometrial cancer rates (160). Since *PTEN* mutations are implicated in many non-MMR spectrum malignancies (such as thyroid), other factors must be included as well to explain why the high penetrance of endometrial cancer in HNPCC, which in women can exceed that of CRC (161,162).

What might explain the susceptibility of uterine endometrium and ovarian epithelium? Recently, it has been proposed that the rate of change of cell proliferation, and not just the rate of proliferation, may play an important role in tumor initiation (163), and both these tissues experience hormonally induced rapid stop/start changes in proliferation rates rather than gradual changes. The endometrium experiences a monthly cycle of estrogen and progesterone responsive abrupt rapid proliferation, followed by abrupt apoptosis and regression. The ovarian epithelium has a similar monthly cycle of hormonally induced rupture to release follicles, and then abrupt rapid proliferation to repair the ruptured epithelium. In summary, these tissues are characterized not only by relatively high proliferation rates but particularly by rapid acceleration and deceleration in the rates of proliferation, which can be characterized by the first derivative of the cell proliferation velocity.

It is noteworthy that stomach and colorectum epithelial cells also are characterized by unusually variable changes in proliferation rate velocity. The classic studies of cell kinetic studies using thymidine labeling of epithelial cells from human stomach, small intestine and colorectum both *in vivo* (164) and *in vitro* short term tissue culture (108,165) show similar overall rates of proliferation in human small intestine, but significantly higher changes in proliferation rate, in human stomach or colorectum epithelium compared with the small intestine (108,164,165). Since the penetrance of stomach and colorectal cancer is consistently much higher than small bowel cancer in HNPCC regardless of geography or diet, these studies provide evidence for a potential link between proliferation rate acceleration/deceleration and the cell types most susceptible to MMR defective mechanisms of carcinogenesis. Furthermore, hematopoietic white blood cell and non-melanotic dermal lineages are susceptible to immunological and (in the latter case) sunlight induced abrupt cycles of stop/start rapid proliferation.

Based on the available data, we propose a model that the hormonally induced cycle of monthly rapid acceleration and deceleration of proliferation rate may play a significant role in uterine endometrial and ovarian epithelial cells to MMR defective cancer susceptibility. If correct, this model would suggest several testable features. First, that endometrial/ovarian cancers rates would be higher with inducible rapid bursts of stop/start proliferation compared to the same number of cell division cycles induced more gradually. Similarly, the model would predict that abruptly blocking endometrial/ovarian epithelial cell hormonal dependent proliferation while controlling for the overall number of cell division cycles would make cells more susceptible to carcinogenesis. Importantly, the model would also predict mechanistically that genes critical for terminating the cell cycle and initiating apoptosis or senescence (stepping on the brakes of proliferation) would be mutated at higher rates in the tissues with rapid bursts of stop/start proliferation compared to those with high proliferation rates but more gradual changes in rates (Figure 1).

This model could first be tested in synchronized cell culture directly using MMR defective cell lines, where mutation rates can be quantified. If there is a proliferative advantage for MMR defective cells in an environment of fast acceleration/deceleration, it would also predict that, analogous to experiments using DNA damaging agents (125), MMR second hit mutations would be selected for by this treatment. Also, it could be tested using MMR-deficient mice and tissue specific inducible oncogenes such as *Myc* that are active only in the presence of specific ligands. The same models could be used to evaluate the affect of rapid acceleration/deceleration of proliferation in tissues with consistently have lower cancer rates in MMR defective individuals such as prostate, breast (60,63,67,117). It is important to note that while hormonally responsive cycles of stop/start proliferation are also seen in breast epithelium, it is distinctive from that seen in the endometrium and ovary. The primary rapid proliferative responses of human breast epithelium are during puberty and pregnancy, not during menses, while the primary hormonally induced response during menses is largely hypertrophic, not hyperproliferative (166,167). If correct, our model would predict in the context of MMR deficiency, pulsatile induction of *Myc* or other oncogenes would have a much greater affect than continuous *Myc* expression.

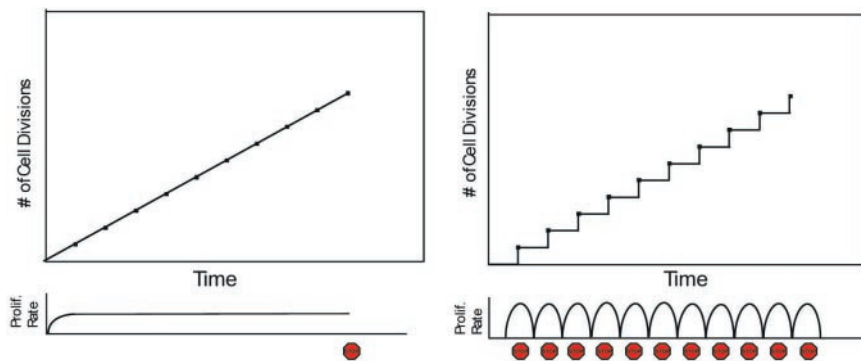


Figure 1. Model of cell proliferation at constant and varying rates of cell division. The left panel shows a conceptual model of cells dividing at a constant rate. The number of cell divisions is plotted on the y-axis and time on the x-axis. The proliferation rate is constant until the tenth cell division, when proliferation stops (as denoted by a Stop sign). The right panel shows a model of cells dividing at varying rates. There are repeated episodes of sharp acceleration and deceleration (as denoted by a Stop sign) of the proliferation rate, starting at time point zero. The proliferation rate is constantly changing and is not constant. The potential selective advantages for growth and increased overall mutations in critical tumor suppressor genes for cells experiencing cycles of abrupt stop/start cell division is discussed in the section 'ACCELERATION and deceleration of proliferation: are the rates of change of proliferation rates important?'

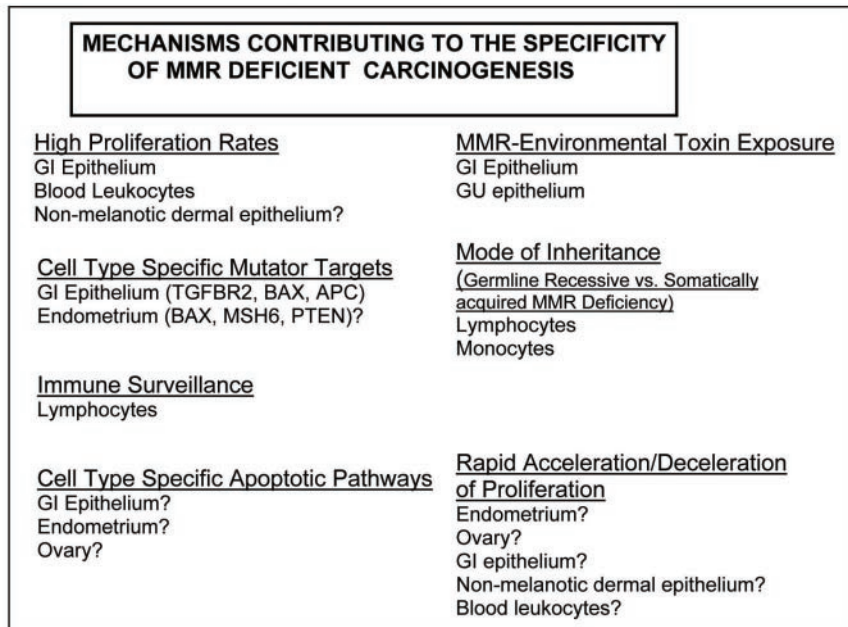


Figure 2. Summary of different mechanisms contributing to the specificity of cell types that are susceptible to MMR deficiency.

CONCLUSION

The tissue specificity of cancers that arise from malfunction of ubiquitously expressed processes such as MMR remains a key puzzle in cancer genetics. Based on the accumulated data, we propose there is likely no single mechanism that can explain fully the cell type specificity observed in the spectrum of MMR defective cancers. A more likely explanation is that several distinct mechanisms contribute to shape the observed tissue specificity, and contribute in different ways to different tissues (Figure 2). For the GI epithelium, important facts include particularly rapid proliferation rates, exposure to mutagens from the diet, the dependence on critical genes whose sequences contain short repetitive sequences. For the endometrium, cell type specific targets of the mutator

and anti-apoptosis functions of MMR are likely important, but endometrium specific targets need to be more precisely characterized. For the renal tract, concentration of environmental mutagens in the urine are likely important, but these mutagens need to be studied in more detail. For the ovary, cell type specific targets are likely to be important, but they too need to be understood more clearly. For lymphocytes, both the mode of inheritance (Germline Recessive versus Somaticall acquired MMR Deficiency) and immune surveillance play important roles. Finally, we propose that not only high proliferation rates, but the rapid acceleration and deceleration of proliferation rates may play a significant role in the specific cell types affected by MMR deficiency, and suggest experiments to test this hypothesis directly.

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