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**REVIEW**

# Carcinogenesis and microsatellite instability: the interrelationship between genetics and epigenetics

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**DNA mismatch repair (MMR) deficiency results in a strong mutator phenotype and high-frequency microsatellite instability (MSI-H), which are the hallmarks of tumors arising within Lynch syndrome. MSI-H is characterized by length alterations within simple repeated sequences, microsatellites. Lynch syndrome is primarily due to germline mutations in one of the DNA MMR genes; mainly *hMLH1* or *hMSH2* and less frequently *hMSH6* and rarely *hPMS2*. Germline hemiallelic methylation of *MLH1*, termed epimutation, has been reported to be a new cause of Lynch syndrome. MSI-H is also observed in ∼15% of colorectal, gastric and endometrial cancers and in lower frequencies in a minority of other tumors, where it is associated with the hypermethylation of the promoter region of *hMLH1*. MSI-H underlies a distinctive tumorigenic pathway because cancers with MSI-H exhibit many differences in genotype and phenotype relative to cancers without MSI-H, irrespective of their hereditary or sporadic origins. Genetic, epigenetic and transcriptomic differences exist between cancers with and those without the MSI-H. The *BRAF* V600E mutation is associated with sporadic MSI-H colorectal cancers (CRCs) harboring *hMLH1* methylation but not Lynch syndrome-related CRCs. The differences in genotype and phenotype between cancers with and those without MSI-H are likely to be causally linked to their differences in biological and clinical features. Therefore, the diagnosis of MSI-H in cancers is thus considered to be of increasing relevance, because MSI-H is a useful screening marker for identifying patients with Lynch syndrome, a better prognostic factor and could affect the efficacy of chemotherapy. This review addresses recent advances in the field of microsatellite instability research.**

## Introduction

A type of genetic instability characterized by length alterations within simple repeated microsatellite sequences, termed high-frequency microsatellite instability (MSI-H), occurs in the majority of Lynch syndrome [hereditary non-polyposis colorectal cancers (HNPCCs)]-associated cancers and in a subset of sporadic cancers (1–4). Genetic and epigenetic inactivation of DNA mismatch repair (MMR) genes leads to mutations in cancer-related genes and to cancer development. MSI-H underlies a distinctive tumorigenic pathway because tumors with MSI-H exhibit many differences in clinical, pathological and molecular characteristics relative to tumors without it, irrespective of their hereditary or sporadic origins. The differences in this can be explained because MMR deficiency leads to an exacerbated mutator phenotype with a very specific mutation spectrum. MSI-H rapidly leads to frameshift mutations in repeated sequences present in target tumor suppressor genes. The peculiar genotype of tumors with MSI-H also includes specific patterns of gene regulation. Gastrointestinal cancers with MSI-H often display an aberrant epigenetic pattern, such as hypermethylation of some genes, including *hMLH1*, the key MMR gene. The differences in the genotype and phenotype between gastrointestinal cancers with and those without MSI-H are likely to be causally linked to their differences in biological and clinical features. Diagnostic characterization of the microsatellite instability (MSI) status, therefore, has implications in clinical oncology. This review summarizes the recent advancements in the knowledge of MSI in gastrointestinal cancers. It will focus on the following topics, multiple alternative genetic pathways to colorectal cancer (CRC), MSI diagnostics, low-frequency MSI (MSI-L), germline epimutation of *hMLH1* and *hMSH2*, relationship between MSI-H and CpG island hypermethylator phenotype (CIMP), *BRAF* mutations and serrated pathway, target genes and the transcriptome of MSI-H tumors.

**Abbreviations:** CIMP, CpG island hypermethylator phenotype; CIMP-H, CIMP-high; CIMP-L, CIMP-low; CIN, chromosomal instability; CRC, colorectal cancer; HDAC, histone deacetylase; HNPCC, hereditary non-polyposis colorectal cancer; LOH, loss of heterozygosity; MMR, mismatch repair; MSI, microsatellite instability; MSI-H, high-frequency MSI; MSI-L, low-frequency MSI; MSS, microsatellite stable; NMD, nonsense-mediated decay; 5-FU, 5-fluorouracil.

### *Genomic instability*

Two genomic instabilities define two distinct pathways for gastrointestinal cancer (1–4). Chromosomal instability (CIN) is associated with the suppressor pathway for aneuploid cancer, and MSI underlies the mutator pathway for (pseudo) diploid cancer. The CIN phenotype is found in ∼85% of sporadic CRCs and is characterized by aneuploidy, multiple chromosomal rearrangements and an accumulation of somatic mutations in oncogenes such as *K-ras* and tumor suppressor genes such as *APC* and *p53*. Recent studies have shown the age-dependent accumulation of DNA hypomethylation (demethylation) to precede genomic damage in a significant subset of gastrointestinal cancers (5,6). The MSI phenotype is found in ∼15% of sporadic CRCs and is associated with small insertions and deletions mainly in repetitive sequences (microsatellites).

In contrast to the widely accepted genetic model of CRC development, only 7 (6.6%) of 106 CRCs were found to contain mutations in all of the *APC*, *K-ras* and *p53* genes and 41 (38.7%) of CRCs had mutations in only one of these genes (7). These results suggest that multiple alternative genetic pathways to CRC exist. The simplest classification of CRC is to divide it into MSI+ and CIN+. However, the molecular mechanisms of genomic instability are not necessarily independent and may not be fully defined by either the MSI or the CIN pathways (8). The near-diploid microsatellite stable (MSS) (MSI− CIN−) group designated as microsatellite and chromosome stable has been identified (9,10). Some MSI− CIN− CRCs and MSI+ CIN+ CRCs appear to form distinct pathways, suggesting that the MSI and CIN pathways are not mutually exclusive in CRC (11). Considerable crosstalk between various pathways is encountered and will be discussed later in this review (4).

### *MSI pathway (cancer as a mutator phenotype)*

Tumors of the Lynch syndrome (HNPCC) and some sporadic gastrointestinal and endometrial cancers belong to the MSI pathway (12,13). MSI accounts for the mutational activation and inactivation of cancer-related genes, those with positive and negative roles in cell growth or survival, which drive multi-step carcinogenesis. Cancers in the MSI pathway accumulate hundreds of thousands of somatic mutations in simple repeated sequences or microsatellites. If the DNA MMR mechanism fails, then spontaneous errors of replication due to

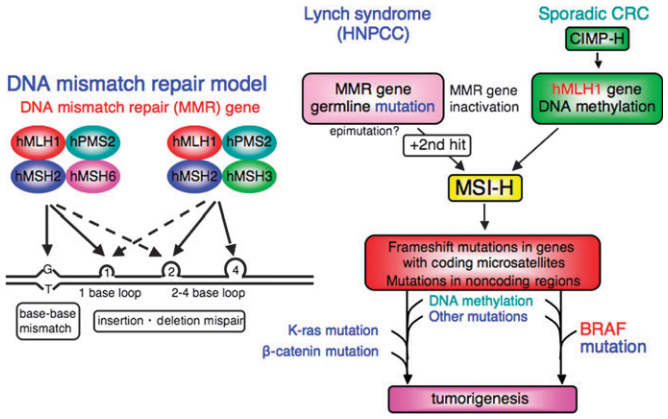


**Fig. 1.** A model of DNA MMR and molecular pathways for CRCs with MSI-H.

slippage by strand misalignment are fixed as mutations and thereafter accumulate because of defects in replication fidelity of these unstable sequences (Figure 1). The discovery of the MSI, by the detection of these ubiquitous somatic mutations, provided conclusive evidence for the hypothesis of cancer as a mutator phenotype (1,2).

*MSI diagnostics*

Diagnostic characterization of the MSI status has implications in clinical settings and oncology, because MSI is a useful screening marker for identifying patients with Lynch syndrome, a better prognostic factor, and could affect the efficacy of chemotherapy (14–16). The standard testing procedure recommended by the National Cancer Institute/International Collaborative Group/HNPCC is the analysis of the tumor and normal tissues by using five microsatellite markers including two mononucleotide repeats (BAT26 and BAT25) and three dinucleotide repeats (D2S123, D5S346 and D17S250) (15,17). Based on the number of markers displaying instability per tumor, three groups of tumors are defined: those with $\geq$30–40% of the markers showing instability (MSI-H); those with $<$30–40% of the markers showing instability (MSI-L) and those showing no instability (MSS).

For the detection of MSI-H cases, BAT26 and BAT25 are the most commonly used mononucleotide markers without the need for corresponding normal tissue. An analysis of BAT26 is sufficient for detecting the MSI-H phenotype in most, but not all, cases (18). However, depending on the ethnic origin of the individuals, short alleles of BAT26 and BAT25 can be seen, which may lead to false-positive results (19). A pentaplex panel of five quasimonomorphic mononucleotide repeats (BAT25, BAT26, NR-21, NR-22 and NR-24) may be more sensitive for MSI-H tumors than other microsatellite markers (19). By analyzing germline DNA from 1206 individuals encompassing 55 different populations worldwide, Buhard *et al.* (20) further demonstrated that MSI can be determined using the pentaplex reaction for all human populations without the need for matching normal DNA.

A new quasimonomorphic marker CAT25, a T25 mononucleotide tract in the 3′ untranslated region of the *caspase 2* gene, has been reported to be monomorphic in normal tissue of individuals of Caucasian, African and Asian origin (21). Findeisen *et al.* (21) concluded that only one polymerase chain reaction amplifying CAT25 alone or multiplexed with BAT25 and BAT26 from tumor DNA is sufficient to yield the same sensitivity and specificity as the five-marker panel recommended by the National Cancer Institute/International Collaborative Group/HNPCC.

Dinucleotide repeats are less sensitive than mononucleotide repeats for the detection of MSI-H. Since the use of dinucleotide repeats may result in under or overestimation of MSI status, the revised Bethesda guidelines mainly recommend the use of more mononucleotide markers in ambiguous situations (only in dinucleotide-unstable cases) (22). Additional results with dinucleotide markers can sometimes lead to an incorrect classification of tumors.

Differences in not only the frequency but also the form of microsatellite alterations are now being considered. Qualitative differences in dinucleotide microsatellite alterations have been reported (23,24). Thibodeau *et al.* (23) and Oda *et al.* (24) divided microsatellite alterations into two categories, Type I and II or Type A and Type B mutations, respectively. A significant expansion or deletion in the fragment size was defined as Type I and 2 bp change was defined as Type II. Length change of $<$6 bp was defined as Type A and length change of $\geq$8 was defined as Type B. MSI-H tumors often exhibit Type I/Type B MSI. p53 mutations, which are uncommon in CRCs with MSI-H or Type B MSI, were frequently associated with Type A instability (24). Oda *et al.* (24) suggest that there are at least two qualitatively distinct modes of dinucleotide MSI in CRC and that different molecular mechanisms may underlie these modes of MSI.

*Lynch syndrome*

Lynch syndrome is best understood as a hereditary predisposition to malignancy caused by a germline mutation in a DNA MMR gene (12,13,25; Figure 1). Predisposed individuals have an increased lifetime risk of developing a variety of cancers including cancers of the colorectum, endometrium, and less frequently, cancers of the small bowel, stomach, urinary tract, ovaries and brain (26–29). Gastric cancer was not included in the Amsterdam II criteria, but it is considered to be associated with Lynch syndrome. Recent molecular analysis combined with the previous demonstration of increased incidence relative to the general population make it justified to consider gastric cancers as true Lynch syndrome spectrum malignancies (30).

Not all families fulfilling the Amsterdam criteria have Lynch syndrome (12). Families fulfilling Amsterdam criteria but who have no evidence of a DNA MMR deficiency do not share the same cancer incidence as families with Lynch syndrome (31). Individuals in such families have a lower incidence of CRC than those in families with Lynch syndrome. Families with a strong family history of CRC that do not have Lynch syndrome have been grouped as 'Familial Colorectal Cancer Type-X' (12). For diagnosis of Lynch syndrome, the Bethesda guidelines should be more sensitive than the Amsterdam criteria and more specific through the inclusion of MMR testing within the algorithm (12,32,33).

Lynch syndrome is caused by germline mutations in DNA MMR genes, predominantly *hMLH1* or *hMSH2*, less frequently *hMSH6* and rarely *hPMS2*, with large genomic rearrangements accounting for 5–20% of all mutations (28,34). *hMSH6* showed a stronger association with endometrial cancer (35). The MSI-H phenotype requires the biallelic 'two hit' inactivation of the responsible MMR gene for tumor development. In Lynch syndrome tumors, somatic inactivation of the remaining wild-type allele can occur due to different mechanisms: loss of heterozygosity (LOH), somatic mutation and promoter methylation. Gene conversion is a frequent mechanism of inactivation of the wild-type allele in cancers from *hMLH1/hMSH2* deletion carriers (34).

Somatic mutations as second hits have been found in both *hMLH1*- and *hMSH2*-deficient tumors, although at low frequencies (36). A loss of the wild-type allele has been detected in 33–86% and thought to be the major mechanism for somatic second hits in most of the studies (36). Using DNA from paraffin-embedded tumor samples, Sanchez de Abajo *et al.* (37) detected frequent loss of the mutant MMR gene allele in Lynch syndrome tumors, proposing a dual role for LOH. However, a recent study using DNA from fresh-frozen tumor samples detected LOH at the wild-type allele, thus supporting the traditional two-hit model of gene inactivation (36).

*hMLH1* methylation has been detected in 0–46% of Lynch syndrome CRCs (36). Frequent (53%) *hMLH1* methylation was seen in Lynch syndrome colorectal adenomas (38). However, methylation assays used in these previous studies are not quantitative. Using a new real-time polymerase chain reaction-based technique to detect and quantify the methylation of both proximal and distal *hMLH1* promoter regions, Bettstetter *et al.* (39) reported that Lynch syndrome CRCs showed no or low level of *hMLH1* promoter methylation. Although the authors concluded that the quantitative *hMLH1* methylation analysis in MSI-H CRC is a valuable molecular tool to

distinguish between Lynch syndrome and sporadic cases, the relevance of monoallelic methylation in Lynch syndrome is not excluded. The patterns of somatic events (LOH and promoter methylation) differ depending on the tissue and germline mutation, which may in part explain the differential tumor susceptibility of different organs in Lynch syndrome (40).

Germline hemiallelic methylation of *hMLH1*, which is termed epimutation, has been reported to be a new cause of Lynch syndrome (41,42). Suter *et al.* (43) advanced the concept by showing vertical transmission of a methylated *MLH1* allele. Hitchins *et al.* (44) reported *hMLH1* germline epimutation in a male subject who had inherited the methylated allele from his mother in whom the same allele was not methylated, suggesting that the epimutation arose as a *de novo* event. Inheritance of a cancer-associated *hMLH1* germline epimutation has been recently reported (45). A heritable germline epimutation of *hMSH2* was also reported in a family with Lynch syndrome (46). Although these findings may represent examples of transgenerational epigenetic inheritance, the possibility of an underlying genetic change that causes heritable methylation cannot be excluded (47).

Lynch syndrome-like CRC families characterized by tumors with variable levels of MSI (MSI variable) among individual members have been reported (48). CRCs and polyps in these families were characterized by a *BRAF* mutation and *MINT31* hypermethylation, thus suggesting an origin in the serrated pathway for CRC, or serrated pathway syndrome (48).

One must also distinguish Lynch syndrome from various diseases, such as attenuated familial adenomatous polyposis, MYH polyposis, juvenile polyposis, germline mutation of *transforming growth factor β receptor II* and *AXIN2*, hereditary mixed polyposis syndrome, hyperplastic polyposis, serrated pathway syndrome, germline hemiallelic methylation of *MLH1*, Lynch syndrome variants and familial CRC-type X (12).

### *Sporadic tumors with MSI-H*

MSI-H also occurs in ∼15% of human colorectal, gastric and endometrial cancers and in lower frequencies in a minority of other tumors (49). Cancers with MSI-H are distributed unequally along the gastrointestinal tract, although the reasons for this asymmetry are not well understood.

CRCs with defective DNA MMR have peculiar molecular, pathologic and clinical characteristics, including MSI-H, poor differentiation, abundant mucin secretion, marked lymphocytic infiltration, preferential location in the proximal colon and better prognosis (12). Sporadic CRCs with MSI-H and Lynch syndrome have similar morphological features. However, mucin secretion, poor differentiation, tumor heterogeneity and glandular serration and co-existing serrated polyps are more evident in sporadic CRCs with MSI-H, whereas lymphocytic infiltration, tumor budding (de-differentiation) and co-existing adenomas are more evident in Lynch syndrome (50).

Gastric cancers with MSI-H are associated with intestinal type, distal location and better survival and these cancers exhibit a significantly lower incidence of *p53* gene mutations than those without MSI-H, thus suggesting that gastric cancers with MSI-H also represent a distinctive oncogenic pathway (51). Pancreatic cancers with MSI-H appear to follow a distinctive oncogenic pathway because they exhibit certain clinical, pathological and molecular characteristics. Pancreatic cancers with MSI-H were associated with poor differentiation, longer overall survival time and the presence of wild-type *K-ras* and *p53* genes (52). In contrast, MSI-H appears to play little, if any, part in hepatocarcinogenesis (53). Type I (endometrioid) cancers are characterized by MSI-H, mutation of phosphatase and tensin homolog deleted in chromosome 10 and *K-ras* and a near-diploid karyotype. Type II (non-endometrioid) cancers are characterized by mutations of *p53* and *Her-2/neu* and non-diploid karyotype (54).

### *hMLH1 methylation and CIMP*

DNA hypermethylation in CpG-rich promoters is now recognized as a common feature of human tumors (55). Cancers can be classified according to their degree of methylation, and those cancers with high degrees of methylation (CIMP) represent a clinically and etiologically distinct group (55,56). Sporadic tumors with MSI-H are associated with the hypermethylation of the promoter region of *hMLH1* (23; Figure 1). Biallelic *hMLH1* promoter methylation is the predominant cause of MSI-H in sporadic tumors, and these tumors rarely show LOH or somatic mutations (36). MSI-H CRCs with methylated *hMLH1* are distinct from the rest by a delayed onset and association with the female gender (57). This finding may explain the previous observations of a higher incidence of MSI-H CRCs in older females (23) because it establishes a link between the female gender and the methylation of the *hMLH1* gene.

There has been considerable overlap between CIMP and MSI-H. CIMP has been postulated to explain silencing of the *hMLH1* gene in cancers with MSI-H (47,48; Figure 1). However, the presence and role of CIMP has been controversial (58–60). Using a large population-based CRC samples ($n = 864$), Samowitz *et al.* (61) determined the biologic relevance of CIMP. Tumors with methylation of more than three of *CDKN2A*, *MINT1*, *MINT2*, *MINT31* and *MLH1* were defined as CIMP-high (CIMP-H). CIMP-H was significantly associated with BRAF and K-ras mutations, older age, advanced tumor stage and proximal colon. Compared with MSS/CIMP-H tumors, MSI-H/CIMP-H tumors were significantly more likely to have *BRAF* mutation and wild-type *K-ras* and *p53* to be poorly differentiated, proximally located and lower stages (Figure 1). MSS/CIMP-H tumors had less intense methylation and *K-ras* mutation. Samowitz *et al.* (61) concluded that MSI-H has a major effect on the expression of CIMP.

According to the systematic, stepwise screening of 195 CpG island methylation markers using MethyLight, Weisenberger *et al.* (62) found that CIMP-positive (CIMP+) tumors convincingly represent a distinct subset, encompassing almost all cases of tumors with a *BRAF* mutation. The mutation of *BRAF* appears to cosegregate with CIMP-H and may be used as a surrogate for CIMP-H (12). Sporadic CRCs with MSI-H occurred almost exclusively as a consequence of CIMP-associated methylation of *hMLH1*. By using expression profiling combined with epigenetic scanning, Mori *et al.* (63) identified genes uniquely methylated in CRC with MSI-H and designated this as MSI-H cancer-specific hypermethylation.

An intrinsic difficulty in establishing whether genetic factors may explain CIMP is the lack of an agreed definition of CIMP (12). A robust new marker panel (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*) to classify CIMP+ (three to five methylated loci) tumors has been proposed (62). More attention needs to be paid to the methodological aspects of methylation analyses and their effects on the results of analyses.

At least two distinct CIMP subsets, CIMP1 (MSI associated) and CIMP2 (non-MSI associated), have been suggested (60). Moreover, CRC with less extensive promoter methylation, designated as 'CIMP-low (CIMP-L)', was reported to be associated with a male sex and *K-ras* mutations (64). An epigenetic defect that influences the spread of methylation from methylation centers has been suggested in CIMP-L. The hypothesis that CIMP-L tumors are different from CIMP-H and CIMP tumors therefore needs to be further investigated.

### *Sporadic MSI-H CRC versus Lynch syndrome*

The mechanisms differ in hereditary (germline mutation) and sporadic (epigenetic silencing) CRCs with MSI-H (Figure 1). While the clear differences in phenotype and genotype of cancers with and those without MSI-H provided the rationale for distinguishing these two pathways for carcinogenesis, hereditary and sporadic CRCs with MSI-H were thought to be similar in their molecular genetic parameters until recently.

The *BRAF* gene encodes a serine/threonine kinase in the RAS/RAF/mitogen-activated protein kinase pathway. *BRAF* mutations play an important role in various types of human malignancy (65). In sporadic CRCs, *BRAF* mutations are more frequently detected in those with MSI-H than those without (66–68). Importantly, an oncogenic V600E hot spot mutation within *BRAF* is frequently detected in

sporadic CRCs with MSI-H but not in Lynch syndrome-related CRCs (69–71; Figure 1). Therefore, *BRAF* V600E mutations may be useful for the screening of Lynch syndrome families. Mutation analysis of the *BRAF* hot spot is a reliable, fast and low-cost strategy that simplifies genetic testing for Lynch syndrome (13,70).

*BRAF* and *K-ras* mutations are mutually exclusive. Distinct patterns of *K-ras* mutations have been reported in CRCs according to germline DNA MMR defects and *hMLH1* methylation status (72). Lynch syndrome, sporadic MSI-H (depending on the *hMLH1* status) and MSS CRCs may target distinct kinases within the RAS/RAF/mitogen-activated protein kinase pathway. Lynch syndrome shares many of the same epigenetic changes as sporadic CRCs with MSI-H, although generally at a low frequency (73,74).

### *Serrated pathway and classification of CRC*

Sporadic CRCs with MSI-H are widely considered to develop from serrated polyps (Figure 2). The recently described sessile serrated adenoma, which differs from classic hyperplastic polyps in having atypical architecture and proliferation (75), has been implicated in the genesis of sporadic CRCs with MSI-H (12,76). A recent prospective study showed that the prevalence of sessile serrated adenomas was ~9% in patients undergoing colonoscopy and were associated with *BRAF* mutation, proximal location, female sex and presence of multiple polyps (77).

Jass (78) has classified CRC based on a correlation of the clinical, morphological and molecular features. Serrated polyps are considered to be the precursors of Type 1 (CIMP-H/MSI-H/*BRAF* mutation) and Type 2 (CIMP-H/MSI-L or MSS/*BRAF* mutation) CRCs. Type 4 (CIMP–/MSS) and Type 5 or Lynch syndrome (CIMP–/MSI-H) may evolve through the adenoma–carcinoma sequence. Type 3 (CIMP-L/MSS or MSI-L/*K-ras* mutation) CRC may arise within either type of polyp.

### *Prognostic significance of MSI-H in CRCs with and without chemotherapy*

MSI-H has been associated with a favorable prognosis. Popat *et al.* (16) reviewed 32 eligible studies stratifying survival in CRC patients by MSI status. They confirmed the relationship between MSI-H and a better survival, with a combined hazard ratio for the overall survival associated with MSI-H of 0.65 (95% confidence interval, 0.59–0.71). On the other hand, the role of MSI-H as a marker to predict a benefit from adjuvant 5-fluorouracil (5-FU) chemotherapy remains contradictory. It has been reported that MSI-H CRC patients either gain a survival advantage from 5-FU chemotherapy or have an extremely good survival when treated with 5-FU (79). On the other hand, Ribic *et al.* (80) reported that the benefits from adjuvant 5-FU chemotherapy may be restricted to MSS CRC patients. Recent retrospective (80,81) and prospective (82,83) studies, in which direct comparisons were made between MSI-H patients with 5-FU chemotherapy and those without, indicate that stage II or III MSS CRC patients benefit from 5-FU chemotherapy, whereas MSI-H CRC patients do not. However, a recent analysis of National Surgical Adjuvant Breast and Bowel Project patients does not support the use of MSI-H as a predictive marker of chemotherapy benefit (84).

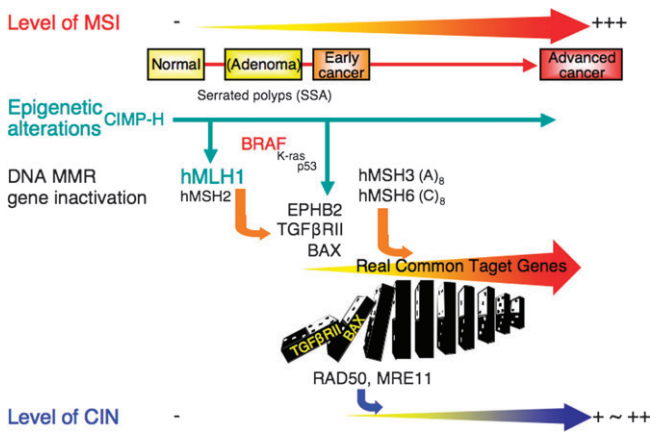


**Fig. 2.** Tumor progression of sporadic CRCs with MSI-H.

Therefore, the currently available data cannot justify the exclusion of MSI-H CRC patients from receiving 5-FU treatment. Randomized clinical trials stratified according to the MSI status comparing 5-FU and other chemotherapeutic drugs should thus be carried out. However, considering the molecular heterogeneity of the MSI phenotype in relation to genetic and epigenetic backgrounds, it may be difficult to use MSI-H as a single predictive marker to guide the use of 5-FU and other chemotherapeutic drugs in CRC patients.

In tumors with MSI-H, MMR deficiency generates many aberrant proteins truncated by frameshift mutations, providing a source of abnormal peptides that can be presented to cytotoxic T lymphocytes. Favorable prognosis may be explained by the marked T cell infiltration seen in MSI-H cancers that is related to a specific antigen-driven immune response (85). By comparing the gene expression profiles of MSI-H and MSS CRCs, Banerjea *et al.* (86) found that many key immunomodulatory genes, such as antigen chaperone molecules, pro-inflammatory cytokines and cytotoxic mediators are up-regulated in MSI-H cancers, suggesting an activated antitumor immune response in these cancers. By means of a transcriptomic expression analysis, Kim *et al.* (87) also found genes that are relevant to the discrimination of MSI-H and MSS CRCs. An intense peritumoral immune response is related to the phenotypic characteristics of MSI-H CRCs (87). These results further support the notion that MSI-H CRCs may be more immunogenic than MSS CRCs (85).

### *Low-frequency MSI*

The presence and role of MSI-L remains controversial (1–4). The nature of MSI-L has not yet been substantiated by characterization of underlying DNA MMR or other defects. Analysis of mutations in target genes for MSI-H revealed that they are absent in MSI-L tumors. Similar results were also obtained in gastric tumors. No significant difference in clinicopathological and molecular variables is observed between MSI-L and MSS CRCs. MSI-L CRCs are indistinguishable from MSS in most parameters. Therefore, these isolated microsatellite alterations are not thought to represent indicators of genomic instability, although they may be useful markers of clonality or mitotic activity (17). The mutations observed in MSI-L cancers may represent a background level of genetic instability present in all cancers and their precursor normal cells (17). If a sufficient number of markers were analyzed, all cancers other than MSI-H cancers would exhibit MSI-L according to the criteria for classification by the National Cancer Institute workshop. MSI-L has been reported to occur in most CRCs (88,89). However, Halford *et al.* (89) found variation in the level of MSI in non-MSI-H CRCs and they suggested that such differences are quantitative and probably reflect the evolutionary histories of the cancers rather than qualitatively different genetic pathways of tumorigenesis.

On the other hand, several reports suggest that MSI-L cancer forms a unique entity. MSI-L is associated with the loss of *O*(6)-methylguanine-DNA methyltransferase protein expression. The loss of *O*(6)-methylguanine-DNA methyltransferase and partial methylation and the loss of the expression of *MLH1* have been suggested to be the mechanisms for MSI-L (77,90). Whitehall *et al.* (90) suggest that silencing of *O(6)-methylguanine-DNA methyltransferase* predisposes genes to mutation by overwhelming the DNA MMR system and occurs with greatest frequency in MSI-L CRCs. MSI-L has been related to frequent instability in the trinucleotide repeat region of *RAS-induced senescence 1* (91) and an independent prognostic factor in stage C CRCs (92). By using the principal components analysis of the complementary DNA microarray-derived data, Mori *et al.* (93) validated the existence of MSI-L tumors as a distinct molecular phenotypic category.

Wu *et al.* (94) have detected four presumably causative *hMSH6* mutations in 4 of 18 patients who had suspected Lynch syndrome and MSI-L cancers. In contrast, Parc *et al.* (95) have found only one somatic *hMSH6* mutation in 41 sporadic cancers with MSI-L, thus suggesting that *hMSH6* mutations do not play a major role in the development of sporadic CRC with MSI-L. Ohmiya *et al.* (96) have reported similar results, thus supporting the role of *hMSH6* as a primary mutator in some hereditary and sporadic cancers but without any correlation between *hMSH6* mutations and MSI-L.

Whether MSI-L cancers may be composed of two groups, a group indistinguishable from MSS cancers and another distinct group that may have a higher number of mutations due to some low or transient instability, remains to be demonstrated. The main problem resides in the difficulty in establishing a criterion for the distinction of these putative 'true' MSI-L cancers from the rest of MSS or false MSI-L cancers based on the number of dinucleotide microsatellite loci alterations in a given set of markers (3). One obvious possibility would be the use of the specificity of mononucleotide repeats instability in MSI-H cancers, and the discovery of any dinucleotide loci that would be exclusively altered in MSI-L cancers but not in MSS cancers.

*Target genes of tumors with MSI-H*

Instability at coding microsatellites in target genes causes frameshift mutations and functional inactivation of affected proteins, thereby providing a selective growth advantage to MMR-deficient cells (97; Figure 3). For instance, *transforming growth factor β receptor II* and the pro-apoptotic gene *BAX* are frequently inactivated by slippage-induced frameshift mutations in mononucleotide tracts present in their gene-coding regions (98,99). These findings are thus considered to provide proof for the causal link between MSI and mutations in cancer-related genes and they are also persuasive examples of the differences between the mutator and suppressor pathways for cancer. These genes have also been mutated in cancers in the suppressor pathway but at fewer frequencies and not by slippage-linked frameshifts (100,101).

The identification of genes relevant for the initiation and/or progression of MSI tumorigenesis is important. Woerner *et al.* (97) analyzed 30 Lynch syndrome-associated MSI-H colorectal adenomas with different grades of dysplasia for frameshift mutations and compared the mutation frequencies with those in MSI-H CRCs. Mutations of several genes, such as *transforming growth factor β receptor II* and *BAX* are detected at high frequencies in the early stages of MSI colorectal tumorigenesis that increased with grade of dysplasia and transition to carcinoma. Frameshift mutations of the receptor tyrosine kinase *EPHB2* are detected more frequently in MSI-H CRCs than in MSI-H colorectal adenomas (102).

A number of cancer-related genes mutated in cancers with MSI-H are being reported (Figure 3). Using an instabilotyping, a large-scale genome-wide screen of coding region microsatellites, Mori *et al.* (103,104) found a number of candidate target genes in gastrointestinal cancers with MSI-H. Mutations in genes carrying microsatellite that promote tumor cell growth are assumed to be the driving force during MSI-H carcinogenesis and designated as Real Common Target genes (49; Figures 2 and 3). On the other hand, microsatellite-harboring genes, mutations of which do not contribute to carcinogenesis, are designated as bystander genes.

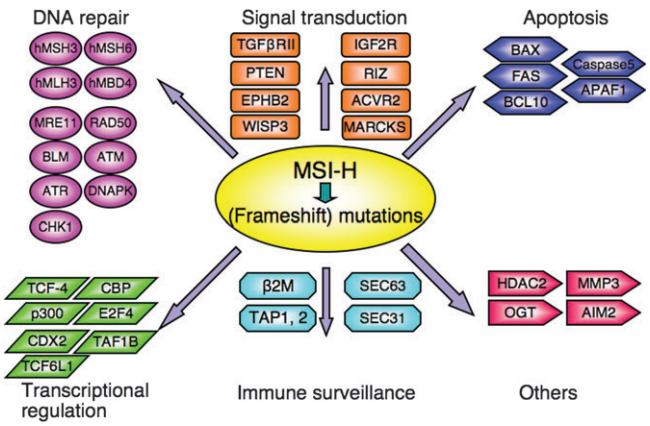


**Fig. 3.** Representative target genes in tumors with MSI-H.

There have been several attempts to find objective criteria for determining mutation functionality based on the stratification of mutation frequency. Duval *et al.* (105) have classified genes into four categories; Transformator (mutations under positive selective pressure), Cooperator, Hibernator, and Survivor (mutations under negative selective pressure) genes. Woerner *et al.* (49) proposed a statistical model that aims to identify Real Common Target genes (106).

However, it is difficult to distinguish genes whose mutations are under positive selection during tumor progression (i.e. relevant, functional mutations) and those that are not (i.e. irrelevant, neutral mutations) based on mutational frequency alone (2). Because MSI-H tumors accumulate many mutations, disruption of cell growth and survival regulation can be accomplished in different tumors by mutations in different genes of the same signaling networks (107). Therefore, genes with infrequent mutations may not be regarded as irrelevant. The relevance of microsatellite-specific mutations in MSI-H tumors can be proven only when there is supporting evidence for functionality, regardless of mutation incidence (2). Nevertheless, Woerner's model is useful for guiding subsequent functional analyses to the most likely target genes among many genes carrying microsatellites.

Tumors with MSI-H demonstrate a high frequency of frameshift mutations that result in the generation of premature translation termination codons. RNA transcripts carrying these nonsense mutations are usually targeted for degradation by the nonsense-mediated decay (NMD) pathway. On the other hand, inhibition of NMD results in stabilization of transcripts carrying nonsense mutations. A strategy referred to as gene identification by NMD inhibition has been developed to identify genes carrying nonsense mutations (108). Stabilized mutant transcripts can be detected on complementary DNA arrays by blocking the NMD pathway with drugs such as emetine. By using the emetine treatment with actinomycin D, which effectively prevents the up-regulation of stress response genes, Ionov *et al.* (109,110) have identified many candidate target genes. By using a further improved strategy, the inhibition of NMD by blocking the phosphorylation of the hUpf1 protein with caffeine, Ionov *et al.* (111) have identified candidate target genes with biallelic mutations.

The target genes of MSI-H tumors can be functionally categorized as tumor suppressors and genes involved in DNA repair, apoptosis and others (Figure 3). Frameshift mutations of *hMSH3* and *hMSH6* led to the concept of 'the mutator that mutates the other mutator' (112). Genomic copy number changes are frequently observed in cancers and they have been demonstrated to contribute to carcinogenesis. It is widely accepted that cancers with MSI-H show less genomic copy number changes and are mostly diploid (113). However, MSI-H tumors could have mutations in genes responsible for CIN and these defects may be selected during tumor progression (114). Indeed, mutations of *hRAD50* and *hMRE11* have been shown to be related to the defects in non-homologous end joining, thus resulting in chromosomal changes during tumor progression (115; Figure 2).

Histone modifications that affect chromatin structures are also implicated in the inactivation of tumor suppressor genes (115). Epigenetic modifier genes could be also the target genes for frameshift mutations. Ropero *et al.* (116) found frameshift mutations of the *histone deacetylase (HDAC) 2* gene in gastrointestinal cancers with MSI-H. *HDAC2* frameshift mutation rendered mutation-positive cancer cells more resistant to the anti-proliferative and pro-apoptotic effects of HDAC inhibitors. Since HDAC inhibitors may serve as therapeutic agents for cancer, these findings support the use of *HDAC2* mutational status in future pharmacogenetic treatment of these individuals (116).

To date, several putative MSI target genes have been proposed mainly based on high mutation frequency within their coding regions. However, some intronic repeat mutations in genes, such as *ATM* and *hMRE11*, have also been suggested to play a role in MSI tumorigenesis (115; Figures 1–3). Non-coding mutations occur not only in introns but also in regulatory regions. The sequences in the promoter region of genes also constitute novel targets of MSI-H tumors. A decreased *matrix metalloproteinase-3* expression due to insertions and/or deletions in the matrix metalloproteinase-3 promoter region resulted in a decrease in the levels of the active matrix metalloproteinase-9 form, which may result in less invasive potential of cancer cells (117). This mechanism, at least in part, may contribute to good prognosis of CRCs with MSI-H (118).

The down-regulation of human leukocyte antigen class I antigen subunits and antigen-processing machinery components are often observed in cancers. In MSI-H tumors, MMR deficiency generates many aberrant proteins truncated by frameshift mutations, providing a source of abnormal peptides that can be presented to cytotoxic T lymphocytes (Figures 1–3). Inactivating mutations in the human leukocyte antigen and *β2-microglobulin* genes, which are required for peptide presentation, is one mechanism by which cancer cells may escape immune recognition by cytotoxic T lymphocytes. Frameshift mutations of the *β2-microglobulin* and antigen-processing machinery genes have been frequently detected in MSI-H gastrointestinal cancers, thus suggesting that these cancers are under selective pressure for obliterating antigen presentation (120–122; Figure 3). Gastrointestinal cancers with and those without MSI-H exhibit differences in inactivation of genes involved in antigen presentation (119–121). Moreover, sporadic MSI-H CRCs and Lynch syndrome have been reported to follow different routes toward the loss of human leukocyte antigen class I expression (122).

Frameshift mutations in coding region could lead to NMD of the RNA transcript. Alterations in the 5′-untranslated region could diminish transcription efficiency and those in 3′-untranslated region could affect RNA stability and processing and translational efficiency (123). Therefore, novel candidate target genes in coding and non-coding regions have been identified by a microarray expression analysis (123). The identified genes of potential relevance for the initiation and progression of MSI tumorigenesis would represent promising candidates for novel diagnostic and therapeutic approaches directed toward MMR-deficient tumors (97; Figures 1–3).

### *Transcriptomic differences between MSI-H and MSS CRCs*

Tumors in the suppressor pathway may derail the homeostatic control of gene expression that is presumably required for tumor development by altering the chromosomal balance. This not only unmasks recessive tumor suppressor genes but also increases the amounts of other cancer gene products with positive roles in cell growth or survival. In contrast, tumors with MSI-H may achieve the same alteration of overall patterns of gene expression by the sheer numbers of frameshift and other (point) mutations.

As molecular markers, gene expression signatures are therefore being developed for many cancers. Array technology has allowed identification of a number of genes that are expressed differentially between MSI-H and MSS CRCs (62,86,87). By using a supervised analysis of the complementary DNA microarray data, Giacomini *et al.* (124) identified a robust expression signature distinguishing MSI and MSS CRC cell lines. By using high-density oligonucleotide microarrays, Kruhoffer *et al.* (125) constructed a gene signature capable of separating CRCs with and those without MSI-H. The authors further constructed a signature capable of separating MSI-H cancers into sporadic and hereditary cases. The identification of a signature for MMR deficiency is therefore considered to be both biologically and clinically relevant (125).

Based on combined mRNA and microRNA gene expression, a molecular signature consisting of 27 differentially expressed genes, including eight microRNAs, has been reported to correctly distinguish MSI-H and MSS CRC samples (126). These results suggest that the combination of mRNA and microRNA expression signatures may represent a general approach for improving the biomolecular classification of human cancer.

## Conclusion

The biological and clinical implications of MSI-H in cancer continue to develop. The clinicopathological, prognostic, genetic, epigenetic and therapeutic characteristics of MSI-H cancers are now becoming clear, but they still remain to be fully elucidated. An analysis of the MSI status in cancer is warranted as a screening for Lynch syndrome, prognostic marker and potential predictive marker of response to chemotherapy. Since molecular-targeting therapeutics are being used in clinical settings and trials, it seems important to clarify whether molecular target genes are differentially regulated between cancers with and those without MSI-H (127). Further analysis is required to gain insight into MSI tumorigenesis, and in order to obtain a better understanding of the pathogenesis, while also developing new therapeutic approaches to target such essential pathogenetic alterations.

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