

## Biallelic inactivation of *hMLH1* by epigenetic gene silencing, a novel mechanism causing human MSI cancers

MARTINA L. VEIGL\*, LAKSHMI KASTURI†, JOSEPH OLECHNOWICZ\*, AIHONG MA\*‡, JAMES D. LUTTERBAUGH†, SUMUDRA PERIYASAMY§, GUO-MIN LI¶, JAMES DRUMMOND||, PAUL L. MODRICH\*\*, W. DAVID SEDWICK\*‡, AND SANFORD D. MARKOWITZ\*†‡††

\*Ireland Cancer Center and ‡Department of Medicine, Case Western Reserve University and University Hospitals of Cleveland, Cleveland, OH 44106; †Howard Hughes Medical Institute, Suite 200, 11001 Cedar Road, Cleveland, OH 44106; §Department of Pharmacology, Medical College of Ohio, 3000 Arlington Avenue, Toledo, OH 43699; ¶Department of Pathology and Laboratory Medicine, University of Kentucky Medical Center, 800 Rose Street, Lexington, KY 40536; ||Biology Department, Indiana University, Third and Hawthorne, Bloomington, IN 47405; and \*\*Howard Hughes Medical Institute, Duke University Medical Center, Naneline Duke Building, Durham, NC 27710

Contributed by Paul L. Modrich, May 21, 1998

**ABSTRACT** Mutations of DNA mismatch repair genes, including the *hMLH1* gene, have been linked to human colon and other cancers in which defective DNA repair is evidenced by the associated instability of DNA microsatellite sequences (MSI). Germ-line *hMLH1* mutations are causally associated with inherited MSI colon cancer, and somatic mutations are causally associated with sporadic MSI colon cancer. Previously however, we demonstrated that in many sporadic MSI colon cancers *hMLH1* and all other DNA mismatch repair genes are wild type. To investigate this class of tumors further, we examined a group of MSI cancer cell lines, most of which were documented as established from antecedent MSI-positive malignant tumors. In five of six such cases we found that *hMLH1* protein was absent, even though *hMLH1*-coding sequences were wild type. In each such case, absence of *hMLH1* protein was associated with the methylation of the *hMLH1* gene promoter. Furthermore, in each case, treatment with the demethylating agent 5-azacytidine induced expression of the absent *hMLH1* protein. Moreover, in single cell clones, *hMLH1* expression could be turned on, off, and on again by 5-azacytidine exposure, washout, and reexposure. This epigenetic inactivation of *hMLH1* additionally accounted for the silencing of both maternal and paternal tumor *hMLH1* alleles, both of which could be reactivated by 5-azacytidine. In summary, substantial numbers of human MSI cancers appear to arise by *hMLH1* silencing via an epigenetic mechanism that can inactivate both of the *hMLH1* alleles. Promoter methylation is intimately associated with this epigenetic silencing mechanism.

Germ-line defects in DNA mismatch repair (MMR) genes account for the inherited familial cancer syndrome of hereditary nonpolyposis colon cancers in which affected individuals show accelerated development of cancers of the proximal colon, endometrium, and stomach (1–5). These cancers typically demonstrate inactivation of the residual wild-type MMR allele inherited opposite the germ-line mutant (1–6), absence of DNA MMR activity in *in vitro* assays (7, 8), and acquisition of an *in vivo* mutator phenotype showing up to 1,000-fold increased gene mutation rates (9, 10). Additionally, these cancers display an associated instability of genomic microsatellite sequences (MSI) (1–5). MSI is similarly found in approximately 15–20% of sporadic colon cancers that arise in individuals without any family history of colon cancer (1–5). Similarly to hereditary nonpolyposis colon cancer-associated

colon cancers, sporadic MSI colon cancers arise predominantly in the proximal colon and show a high rate of frameshift mutations at a mutational hotspot in the transforming growth factor- $\beta$  type II receptor tumor suppressor gene (1, 11). Familial and sporadic MSI colon cancers thus appear to share a common carcinogenic pathway. In this regard, previous studies from our group established that MMR gene inactivation via somatic mutation was the cause of some cases of sporadic MSI colon cancers (12). However, unexpectedly, in many sporadic MSI colon cancers, MMR genes were found to remain wild type (12). MMR-coding sequences have similarly been reported to be wild type in most sporadic MSI endometrial cancers (13). Thus, a mechanism that induces MSI independently of MMR gene mutation appears to account for a significant number of human MSI cancers. Intriguingly, methylation of the *hMLH1* promoter region has recently been described in some MSI tumors (14).

In this study we focused on further elucidating the mechanism that results in MSI in sporadic cancers bearing apparently wild-type MMR genes. We report here that, in five sporadic MSI cancers (four colon and one endometrial) in which MMR genes are wild type, MSI arises via a new epigenetic mechanism that inactivates MMR function by silencing transcription of the wild-type *hMLH1* MMR gene. Epigenetic inactivation of *hMLH1* is biallelic, is associated with methylation of the *hMLH1* promoter, and is reversed by the demethylating agent 5-azacytidine. Moreover, *hMLH1* inactivation is reestablished after withdrawal of the demethylating agent, suggesting that an underlying active process maintains the inactivated methylated state.

### MATERIALS AND METHODS

**Cell Culture.** Cells were cultured in MEM supplemented with gentamycin, L-glutamine, nonessential amino acids, and sodium selenite. AN3CA growth medium was supplemented with 10% fetal bovine serum and sodium pyruvate. For HCT116, Vaco5, and Vaco6, growth medium was supplemented with 8% calf serum. For SW480, RKO, Vaco432, Vaco457 and Vaco703, growth medium was supplemented with 2% fetal bovine serum (HyClone), 10 mg/liter bovine insulin, 2 mg/liter human transferin, and 1 mg/liter hydrocortisone. Monolayer cultures were grown at 37°C in a 5% CO<sub>2</sub> atmosphere. The Vaco cell lines were established from colon cancers as described (11, 15). HCT116, AN3CA, and SW480 were obtained from the American Type Culture Collection,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1998 by The National Academy of Sciences 0027-8424/98/958698-5\$2.00/0  
PNAS is available online at <http://www.pnas.org>.

Abbreviations: MMR, mismatch repair; MSI, microsatellite instability.  
††To whom reprint requests should be addressed at: U.C.R.C. #2,  
Room 200, 11001 Cedar Road, Cleveland, OH 44106. e-mail:  
sxm10@po.cwru.edu.

and RKO was a generous gift from M. Brattain (University of Texas Health Science Center, San Antonio, TX).

**5-Azacytidine Treatment.** Cells were seeded at  $10^5$  cells per T75 flask on day 0. The cultures were treated for 24 h on day 2 and day 5 with 5-azacytidine at 1–3  $\mu\text{g}/\text{ml}$  as individually tolerated. The medium was changed 24 h after addition of the 5-azacytidine (i.e., on day 3 and day 6). On day 8 the cells were harvested for analysis of the methylation status of the *hMLH1* promoter region and of production of hMLH1 protein. Additionally, in some experiments on day 8 cells were either reseeded for continued analysis of hMLH1 expression in bulk culture or subcloned by limiting dilution on 96-well culture plates.

**Evaluation of Methylation Status of the *hMLH1* Promoter Region.** The methylation status of the *hMLH1* promoter was evaluated by a PCR-based assay according to the procedure detailed by Kane *et al.* (14). Briefly, genomic DNA samples were digested with no enzyme, *HpaII*, or *MspI*, and then the 603-bp region upstream of the *hMLH1* gene, which contains four *HpaII* sites, was amplified by PCR. The amplification products were then visualized with ethidium bromide after agarose gel electrophoresis.

**Western Analysis.** Approximately  $10^7$  cells were lysed in cell lysis buffer [50 mM Tris-HCl (pH 7.4)/1 mM EGTA/1% Nonidet P-40/0.25% sodium deoxycholate/150 mM NaCl] and were subjected to electrophoresis through an SDS polyacrylamide gel. The resultant proteins were then transferred to a poly(vinylidene difluoride) nylon membrane, which was probed with 2  $\mu\text{g}/\text{ml}$  anti-human MLH1 monoclonal antibody (PharMingen). Immune complexes were visualized by using an enhanced chemiluminescence reagent (Amersham) after incubation with horseradish peroxidase-coupled secondary antibody (Calbiochem). The membrane was then deprobed and reprobed with 300 ng/ml anti-human actin monoclonal antibody (Amersham).

**Amplification and Sequencing of *hMLH1* Genomic and cDNA.** Genomic DNA was obtained by lysing cells in proteinase K buffer [10 mM Tris (pH 7.4)/10 mM EDTA/0.4% SDS/150 mM NaCl/100  $\mu\text{g}/\text{ml}$  proteinase K] and extracting with phenol/chloroform/isoamyl alcohol (25:24:1). Genomic DNA was amplified in a thermocycler for 38 cycles by using the following protocol: cycle 1,  $95^\circ\text{C} \times 5$  min; subsequent cycles,  $95^\circ\text{C} \times 45$  s,  $55^\circ\text{C} \times 30$  s, and  $72^\circ\text{C} \times 30$  s; final cycle,  $72^\circ\text{C} \times 5$  min and hold at  $4^\circ\text{C}$ . Each 30- $\mu\text{l}$  reaction contained 100 ng genomic DNA, 0.125 mM 2'-deoxynucleoside 5'-triphosphate, 300 ng forward primer, 300 ng reverse primer, and 2.5 units Perkin-Elmer Amplitaq Gold polymerase.

Cells were treated with guanidine isothiocyanate, and RNA was isolated by sedimentation through cesium chloride as described (16). The cDNA for *hMLH1* was amplified by using the Titan RT-PCR system (Boehringer Mannheim). Reverse transcription was carried out for 30 min at  $50^\circ\text{C}$  and then by 38 cycles of PCR (cycle 1,  $95^\circ\text{C} \times 2$  min; subsequent cycles,  $95^\circ\text{C} \times 45$  s,  $54^\circ\text{C} \times 45$  s, and  $68^\circ\text{C} \times 1$  min; final cycle,  $68^\circ\text{C} \times 7$  min and hold at  $4^\circ\text{C}$ ).

The primer sequences were chosen by using MACVECTOR 5.0/2 (International Biotechnology, New Haven, CT) software and the following GenBank *hMLH1* sequence accession numbers: U83845 for the promoter, U07418 for the cDNA, and U40967 for exon 8. A 217-bp genomic fragment encompassing *hMLH1* codon 219 was amplified with primers MLHEX8F1 (5'-CTCAGCCATGAGACAATAAATCC) and MLHEX8R1 (5'-GGTTCCCAAATAATGTGATGG). An *hMLH1* cDNA fragment encompassing codon 219 and spanning exons 7–9 was amplified with primers MLH564S (5'-CAGGTATT-CAGTACACAATGCAGGC) and MLH823AS (5'-CTACAGACGATGGTTGATGAAGAG). The putative *hMLH1* promoter region extending from 765 bp 5' to 57 bp 3' of the ATG was amplified with primers MLHF5 (5'-CACGAGCA-GTCTCTCTTCAGGAGT) and MLHR5 (5'-GATGCGGT-

TCACCACTGTCTCG). Sequencing of the codon 219 polymorphism was done with primer MLHEX8F1 in genomic DNA samples and primer MLH564S in cDNA samples. Sequencing primers for the *hMLH1* promoter region are available from the authors by request.

## RESULTS

**Loss of MLH1 Protein Expression and Functional Activity in Sporadic MSI Cancers.** We initially investigated five cell lines established from sporadic MSI colon cancer tumors that we had previously reported bore only wild-type MMR genes (Vaco432, Vaco5, Vaco6, RKO, and Vaco481) (12). Fig. 1 depicts our Western blot analysis that demonstrates absence of hMLH1 protein expression in four of these five cell lines (Vaco432, Vaco5, Vaco6, and RKO). Furthermore, the western blot shows that no hMLH1 protein was expressed in a sporadic MSI endometrial cancer, AN3CA, which previously was shown to possess wild-type MMR genes but to lack *hMLH1* transcript (17). Finally, hMLH1 protein was also absent in the Vaco703 colon cancer, which we previously reported carries an inactivating somatic mutation of the *hMLH1* gene (12), and in Vaco457, a hereditary nonpolyposis colon cancer-derived colon cancer. Expression of hMLH1 protein was, however, readily detected in the non-MSI colon cancer SW480. Northern analysis confirmed that, in sporadic MSI cancers with wild-type *hMLH1*-coding sequences, those lacking hMLH1 protein expression also lacked hMLH1 transcript expression. Western blots demonstrated continued expression of hMSH2 protein in Vaco5, Vaco6, Vaco432, RKO, and AN3CA. This suggested that in all of these cases the expression of the wild-type *hMLH1* alleles had been specifically silenced.

To characterize the functional consequences of the observed loss of detectable hMLH1 protein, MMR activity was assayed in nuclear extracts derived from Vaco6, RKO, and AN3CA. *In vitro* experiments, to be described elsewhere, indicated that Vaco6, RKO, and AN3CA have an MMR defect caused by an MutL $\alpha$  deficiency similar to that observed previously with cisplatin- and adriamycin-resistant ovarian tumor cells (18). Functional repair activity could be restored by addition of hMutL $\alpha$ , the heterodimeric protein complex of hMLH1 and hPMS2, to each of these extracts (19). Thus, the absence of hMLH1 protein expression in these cancers is the direct cause of their MSI phenotype.

**Loss of *hMLH1* Expression Is Associated with Methylation of the *hMLH1* Gene Promoter.** To establish the mechanism of loss of *hMLH1* gene expression in AN3CA, RKO, Vaco5, Vaco6, and Vaco432, we first sequenced the *hMLH1* promoter for evidence of mutations. No mutations were found in any of these cell lines within 685 bp 5' of the *hMLH1* initiator ATG. We next examined the *hMLH1* promoter region in these tumors for evidence of methylation at four *HpaII* sites located between 341 and 567 bp 5' of the *hMLH1* ATG (14). Meth-

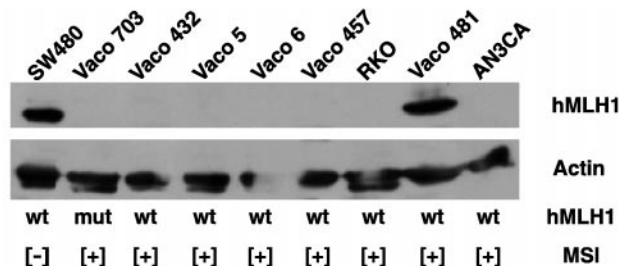


FIG. 1. hMLH1 protein expression in MSI cancers. Shown is a Western blot of hMLH1 protein expression. The presence (+) or absence (-) of MSI and the status of the coding region of the *hMLH1* gene (wt, wild type; mut, mutant) are denoted below each lane. The Western analysis was performed as described in *Materials and Methods*.

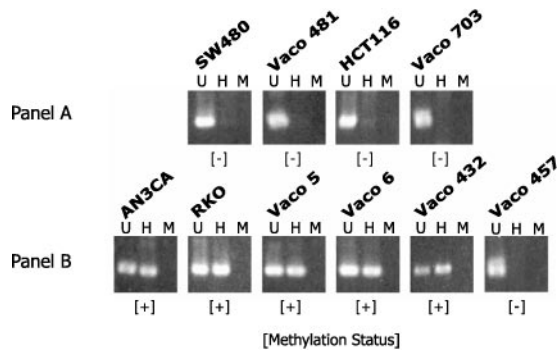


FIG. 2. Analysis of methylation status of the *hMLH1* promoter. Shown is the product resulting from PCR amplification of the *hMLH1* promoter region before or after digestion. U, Undigested; H, digested with *HpaII*; M, digested with *MspI*. The methylation status (+, methylated; -, unmethylated) of the *hMLH1* promoter is designated below each sample.

ylation was detected by the ability to recover and amplify an intact *hMLH1* promoter fragment after digestion of genomic DNA with the methylation-sensitive restriction enzyme *HpaII*. As shown in Fig. 2B, the five sporadic MSI cancers that displayed loss of *hMLH1* expression also have methylation of the *hMLH1* promoter (AN3CA, RKO, Vaco5, Vaco6, and Vaco432).

In contrast to *hMLH1* promoter methylation in MSI cancers with wild-type MMR genes, the *hMLH1* promoter was found to be unmethylated in two MSI cancers in which *hMLH1* protein was inactivated by mutation (HCT116 and Vaco703; Fig. 2A), in one MSI cancer in which *hMLH1* protein was inactivated by an unknown germ-line defect (Vaco457; Fig. 2B), and in two cancers in which wild-type *hMLH1* gene expression was maintained (Vaco481 and SW480). Methylation of the *hMLH1* promoter was thus associated with sporadic MSI cancers that lacked MMR gene mutations.

***hMLH1* Silencing Is Reversible After Treatment with the Demethylating Agent 5-Azacytidine.** To determine whether *hMLH1* promoter methylation could be further linked to the loss of *hMLH1* expression, cancer cell lines were treated with the demethylating agent 5-azacytidine. As shown in Fig. 3, 5-azacytidine successfully restored *hMLH1* protein expression in each of the five sporadic MSI cancers that lacked *hMLH1* expression and that harbored a methylated *hMLH1* promoter. In contrast, 5-azacytidine was ineffective in reactivating *hMLH1* expression in two MSI cancers in which the *hMLH1* promoter was unmethylated and in which *hMLH1* inactivation was caused by somatic mutation (Vaco703) or germ-line defect (Vaco457) (Fig. 3). Thus, the presence of *hMLH1* promoter methylation was tightly correlated with the ability to reactivate *hMLH1* by 5-azacytidine treatment. Moreover, the 5-azacytidine induction of *hMLH1* expression in five sporadic MSI cancers repeatedly demonstrated that an epigenetic mechanism must be responsible for silencing of *hMLH1* expression.

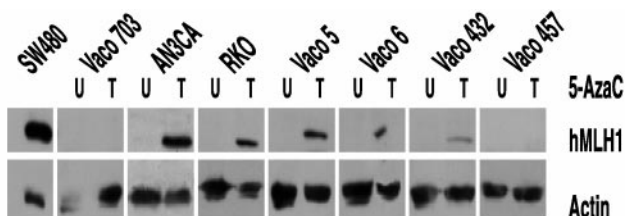


FIG. 3. Induction of *hMLH1* protein expression after 5-azacytidine (AzaC) treatment. The Western analysis was performed as described in *Materials and Methods*. U, Cells untreated; T, cells treated with 5-azacytidine.

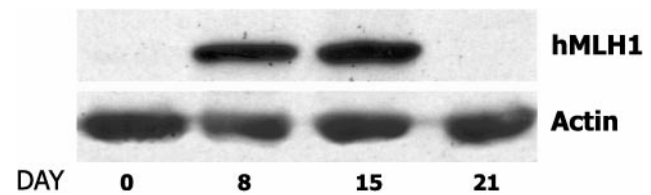


FIG. 4. Time course of *hMLH1* protein expression after 5-azacytidine treatment. AN3CA cells were treated with 5-azacytidine as detailed in *Materials and Methods*. An untreated sample (day 0), a sample immediately after 5-azacytidine treatment (day 8), and samples 1 week (day 15) and 2 weeks (day 21) after 5-azacytidine treatment were collected for Western analysis to evaluate production of *hMLH1* protein.

***hMLH1* Expression Is Again Silenced After Withdrawal of 5-Azacytidine.** To determine whether *hMLH1* gene silencing is actively maintained, we asked whether *hMLH1* gene reexpression would persist after washout of the 5-azacytidine-inducing agent. As shown in Fig. 4, 5-azacytidine treatments of AN3CA cells on days 2 and 5 induced abundant *hMLH1* protein expression by day 8. However by day 21, 2 weeks after drug washout, *hMLH1* protein expression was again undetectable. This result suggested that, after withdrawal of 5-azacytidine, an active process again resiled expression of the *hMLH1* gene.

Alternatively, during the 14 days after drug washout the AN3CA culture could be overgrown by a subpopulation of cells resistant to 5-azacytidine. To distinguish between these two possibilities, we reprised the 5-azacytidine treatment of AN3CA with the addition that, after withdrawal of the 5-azacytidine, 22 independent single-cell subclones were immediately established from the AN3CA culture. As shown in Fig. 5, in this repeat study 5-azacytidine again reinduced *hMLH1* protein expression in the AN3CA cancer cells by day 8. However, in each of the 22 subclones established from the drug-treated population, washout of the drug again was followed by *hMLH1* silencing. To avoid any selection bias favoring subcloning of a putative rapidly growing 5-azacytidine-resistant cell population, we included in this analysis subclones that grew either rapidly or slowly after 5-azacytidine withdrawal. Furthermore, retreating 10 of these subclones with 5-azacytidine in each case resulted in reexpression of the *hMLH1* protein (Fig. 5), demonstrating that the subclones indeed were comprised of 5-azacytidine-responsive cells. Expression of *hMLH1* in AN3CA could thus be cycled from off, to on, to off, and to on, by 5-azacytidine treatment, washout, and retreatment. These findings clearly establish that inactivation of *hMLH1* expression in AN3CA is caused by an epigenetic process. Moreover, these findings suggest that

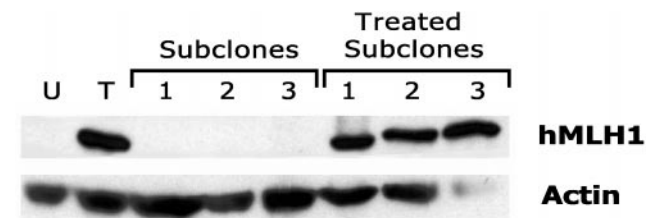


FIG. 5. Reversible expression of *hMLH1* protein in single-cell clones treated with 5-azacytidine. Western analysis of *hMLH1* protein expression was performed as described in *Materials and Methods* and is shown for AN3CA cells untreated (U) or on day 8 of treatment with 5-azacytidine (T). As described in *Materials and Methods*, subclones 1, 2, and 3 were established from the day 8 treated AN3CA cell population immediately after washout of the drug and were analyzed for continued *hMLH1* expression when they had reached confluence. The same three subclones were then retreated with 5-azacytidine and again analyzed (treated subclones). Subclones 1 and 3 are representative normal-growth-rate subclones; whereas subclone 2 is representative of the group of slow-growth-rate subclones.

*hMLH1* silencing is maintained by an active mechanism that is reasserted after washout of the 5-azacytidine inducing agent.

**Epigenetic Inactivation of *hMLH1* Is Biallelic.** No wild-type *hMLH1* protein was detected by Western analysis in the Vaco5, Vaco6, Vaco432, RKO, or AN3CA cell lines, demonstrating in these tumors that both wild-type *hMLH1* alleles had been inactivated. In many cancers biallelic inactivation of suppressor genes results from mutation of one allele and then by gene deletion of the remaining allele (1). This two-step process is reflected by loss of heterozygosity of polymorphic markers linked to the suppressor gene locus (1). We looked for such loss of heterozygosity at the *hMLH1* locus in three of the cases, Vaco5, Vaco6, and Vaco432, for which matched normal tissue was available. A coding region polymorphism at codon 219 (ATC to GTC) was found to be informative in two cases, Vaco5 and Vaco432 (12). Significantly, retention of both maternal and paternal alleles was detected in each of the Vaco5 and Vaco432 tumors and cell lines. Moreover, after 5-azacytidine treatment, induction of transcripts bearing both maternal and paternal polymorphisms was detected in cDNA amplified from either Vaco5 or Vaco432 (Fig. 6). Thus, in these tumors biallelic inactivation of *hMLH1* expression is caused by epigenetic inactivation of both parental copies of the gene. This finding suggests that in these tumors epigenetic silencing of *hMLH1* is not a rare chance event but the end result of an active and efficient mechanism that can work in *trans*. Furthermore, material was available to confirm that the antecedent tumor from which the Vaco432 cell line was established demonstrated both the MSI phenotype as well as methylation of the *hMLH1* gene promoter. Thus, epigenetic inactivation of *hMLH1* almost certainly was the initiating event in the genesis of the MMR deficiency of this individual's colonic malignancy.

## DISCUSSION

These findings establish that the epigenetic silencing of the *hMLH1* gene promoter is an important event in many sporadic human colon cancers. This epigenetic silencing mechanism is closely associated with methylation of the *hMLH1* gene promoter. Moreover, this mechanism was operative in a majority, four of seven, of the sporadic MSI colon cancers in our collection. Additionally, in three of these four cases, we confirmed that the MSI phenotype was present in the antecedent tumors from which the cell lines were established. Thus, the mechanism accounting for MSI in the cell lines almost certainly was present in the antecedent primary tumors. Although larger confirmatory trials will be necessary, our findings suggest that epigenetic inactivation of *hMLH1* underlies the pathogenesis of many human MSI colon cancers.

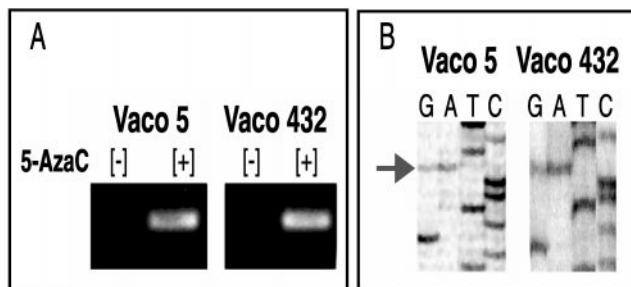


FIG. 6. Allele-specific analysis of *hMLH1* expression after 5-azacytidine treatment in Vaco 5 and Vaco 6. (A) Reverse transcription-PCR analysis of *hMLH1* expression in Vaco5 and Vaco432 before (+) and after (-) treatment with 5-azacytidine (AzaC). (B) DNA sequences of *hMLH1* cDNAs from Vaco5 and Vaco432 amplified by reverse transcription-PCR after treatment with 5-azacytidine. The arrow points to a coding region polymorphism at codon 219 (ATC to GTC).

Colon cancer has served as a useful paradigm for understanding the molecular mechanisms of human carcinogenesis. In many colon cancers biallelic inactivation of two tumor suppressor genes, APC and p53, is demonstrable (1), and for these genes biallelic inactivation is usually caused by mutational inactivation of one allele, accompanied by deletion or loss of the cognate wild-type allele via mechanisms that cause loss of heterozygosity (1). In contrast, we found biallelic inactivation of the *hMLH1* MMR gene commonly proceeds by epigenetic inactivation of both parental alleles and is not accompanied by loss of heterozygosity. These findings suggest that in these cancers epigenetic gene inactivation is a highly efficient method for silencing *hMLH1* gene expression.

Previous studies of MSI colon cancers have demonstrated that these cancers as a class harbor increased methylation within promoter regions of multiple genes, including increased methylation of a candidate tumor suppressor gene, *p16*, as well as of a candidate tumor-promoting growth factor, the insulin-like growth factor II (20). It is thus possible that a large subset of MSI tumors harbors a general defect in gene regulation that induces aberrant promoter methylation at many loci. In this instance methylation and silencing of the *hMLH1* promoter would provide a direct route that connects a general defect in promoter regulation with the specific MSI-associated pathway of carcinogenesis. This would be similar to the finding that mutational inactivation of a tumor suppressor gene, the type II transforming growth factor- $\beta$  receptor, links the global mutator phenotype accompanying MSI with a defined pathway of malignant transformation (9–11). Future studies should reveal the specific relationship between epigenetic inactivation of the *hMLH1* promoter and increased promoter methylation seen at other loci in some MSI cancers.

In addition to showing increased methylation of endogenous promoters, MSI cancers also have an increased propensity to methylate and silence exogenous retroviral gene sequences (21). This phenotype has most clearly been demonstrated for the HCT116 MSI colon cancer in which the *hMLH1* gene is inactivated by mutation and in which the *hMLH1* gene promoter is unmethylated. Future studies should elucidate whether the propensity to methylate exogenous gene sequences is or is not a reflection of the same disordered mechanism that underlies methylation and gene silencing of the *hMLH1* locus. Additionally, increased methylation of the estrogen receptor promoter in normal colonic tissue has been reported to be a correlate of aging (22). Again, elucidating the relationship between this form of aberrant methylation and the epigenetic inactivation of the *hMLH1* gene in cancer will be an important question for future investigation.

The causal role of MMR gene inactivation in initiating the pathway of MSI colon carcinogenesis suggests that in the tumors we studied the methylation and epigenetic silencing of the *hMLH1* gene were neither consequences of disordered gene regulation in cancer nor results of the establishment of cancer cell lines. Instead, the epigenetic silencing of *hMLH1* was a key pathophysiologic step in the genesis of these cancers. These findings support the idea that a similarly direct role in carcinogenesis is played by other tumor suppressor genes, such as *p16* and *VHL*, in cancers in which those genes have been noted to be methylated and to additionally be inducible by 5-azacytidine (23–26).

Methylation of the *hMLH1* gene promoter in association with absent *hMLH1* protein was previously noted in several MSI colon cancers and in the AN3CA endometrial cancer (14). This current study establishes that *hMLH1* promoter region methylation is strongly associated with epigenetic gene silencing, that the silencing mechanism is sufficient to accomplish biallelic inactivation of the *hMLH1* gene, that silencing of the *hMLH1* gene is reversible, that silencing of the *hMLH1* gene appears to be actively maintained, and that *hMLH1* silencing is a common route for the genesis of sporadic MSI cancers.

Additionally, while this study was in progress, Herman *et al.* (27) also noted a significant incidence of *hMLH1* promoter methylation among sporadic MSI colon cancers, as well as the ability to induce *hMLH1* expression with demethylating agents. The large number of tumors analyzed in these three studies provides strong and consistent support for the contention that epigenetic silencing of *hMLH1* is a common route leading to the genesis of sporadic MSI colon cancers. Also in support of this contention is the recent report that *hMLH1* protein expression is absent by immunohistochemical assay in 90% of unselected human MSI colon cancers (28).

A singular finding of this current study is the observation that epigenetic inactivation of *hMLH1* is biallelic and that reactivation of the *hMLH1* allele is rapidly followed by re-silencing of the gene. These observations suggest that *hMLH1* silencing is the result of an underlying active mechanism that efficiently turns off *hMLH1* gene expression. Moreover, it is highly tempting to suggest that *hMLH1* promoter methylation is the direct mechanism that inactivates *hMLH1* gene expression. However, both hypotheses require further investigation, and final judgment will have to await the ultimate elucidation of the mechanisms mediating *hMLH1* promoter methylation and *hMLH1* gene silencing.

Current data do not allow us to judge whether reexpression of wild-type *hMLH1* will have therapeutic utility in certain MSI colon cancers, e.g., by reversing the resistance of such tumors to treatment with certain alkylating agents (29). Any trial of such therapy will need to reflect our finding that repetitive dosing of 5-azacytidine is required to maintain *hMLH1* expression. It is also tempting to consider that demethylating agents might play a role in cancer prevention in individuals who are at risk for cancer or in individuals in whom *hMLH1* promoter methylation might be detected as an early neoplastic change. Our data support the suggestion that *hMLH1* gene silencing accounts for a significant number of human colon cancers. Additionally, the epigenetic inactivation of *hMLH1* demonstrated in the AN3CA MSI endometrial cancer may prove to be representative of MSI endometrial cancers, which as a class bear mostly wild-type MMR alleles (13). Finally, many sporadic human gastric cancers also arise via the MSI pathway (2). Elucidating the ultimate mechanism underlying the epigenetic inactivation of *hMLH1* expression may thus prove relevant to the understanding of many different types of human cancer.

We thank James K. V. Willson for establishing the Vaco cell lines and Bert Vogelstein for his help in determining the MSI status and the sequences of the MMR genes in the Vaco cell lines and also for many helpful discussions. We also thank James Herman and Bo Liu for helpful discussions and Jen Sedwick for help with manuscript preparation. This work was supported by Grant CA 67409 (to S.D.M.), Grant CA 72160 (to S.D.M.), Grant CA 70788 (to M.L.V.), and Grant CA 4370301 (to the Case Western Reserve University Cancer Center) from the National Institutes of Health, Grant FRA-451 (to S.D.M.) from American Cancer Society, Grant 96B084 (to W.D.S.) from American Institute for Cancer Research, and from funds from the State of Ohio Board of Regents. S.D.M. and P.M. are Investigators of the Howard Hughes Medical Institute.

1. Kinzler, K. & Vogelstein, B. (1996) *Cell* **87**, 159–170.
2. Eshleman, J. & Markowitz, S. (1995) *Curr. Opin. Oncol.* **7**, 83–89.

3. Marra, G. & Boland, C. R. (1995) *J. Natl. Cancer Inst.* **87**, 1114–1125.
4. Kolodner, R. (1996) *Genes Dev.* **10**, 1433–1442.
5. Perucho, M. (1996) *Biol. Chem.* **377**, 675–684.
6. Leach, F., Nicolaides, N., Papadopoulos, N., Liu, B., Jen, J., Parsons, R., Peltomäki, P., Sistonen, P., Aaltonen, L., Nyström-Lahti, M., *et al.* (1993) *Cell* **75**, 1215–1225.
7. Parsons, R., Li, G.-M., Longley, M. J., Fang, W. H., Papadopoulos, N., Jen, J., de la Chapelle, A., Kinzler, K. W., Vogelstein, B. & Modrich, P. (1993) *Cell* **75**, 1227–1236.
8. Modrich, P. & Lahue, R. (1996) *Annu. Rev. Biochem.* **65**, 101–133.
9. Eshleman, J., Lang, E., Bowerfind, G., Parsons, R., Vogelstein, B., Willson, J., Veigl, M., Sedwick, W. D. & Markowitz, S. (1995) *Oncogene* **10**, 33–37.
10. Bhattacharyya, N., Skandalis, A., Ganesh, A., Groden, J. & Meuth, M. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 6319–6323.
11. Markowitz, S., Wang, J., Myeroff, L., Parsons, R., Sun, L., Lutterbaugh, J., Fan, R., Zborowska, E., Kinzler, K., Vogelstein, B., *et al.* (1995) *Science* **268**, 1336–1338.
12. Liu, B., Nicolaides, N., Markowitz, S., Willson, J., Parsons, R., Jen, J., de la Chapelle, A., Hamilton, S., Kinzler, K. & Vogelstein, B. (1995) *Nat. Genet.* **9**, 48–53.
13. Katabuchi, H., van Rees, B., Lambers, A. R., Ronnett, B. M., Blazes, M. S., Leach, F. S., Cho, K. R. & Hedrick, L. (1995) *Cancer Res.* **55**, 5556–5560.
14. Kane, M., Loda, M., Gaida, G., Lipman, J., Mishra, R., Goldman, H., Jessup, J. M. & Kolodner, R. (1997) *Cancer Res.* **57**, 808–811.
15. Willson, J., Bittner, G., Oberley, T., Meisner, G. & Weese, J. (1987) *Cancer Res.* **47**, 2704–2713.
16. Davis, L., Dibner, M. & Battey, J. (1986) *Basic Methods in Molecular Biology* (Elsevier Science, New York), p. 388.
17. Boyer, J. C., Umar, A., Risinger, J. I., Lipford, J. R., Kane, M., Yin, S., Barrett, J. C., Kolodner, R. D. & Kunkel, T. A. (1995) *Cancer Res.* **55**, 6063–6070.
18. Drummond, J., Anthony, A., Brown, R. & Modrich, P. (1996) *J. Biol. Chem.* **271**, 19645–19648.
19. Li, G.-M. & Modrich, P. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 1950–1954.
20. Ahuja, N., Mohan, A., Li, Q., Stolker, J., Herman, J., Hamilton, S., Baylin, S. & Issa, J.-P. (1997) *Cancer Res.* **57**, 3370–3374.
21. Lengauer, C., Kinzler, K. & Vogelstein, B. (1997) *Proc. Natl. Acad. Sci. USA* **94**, 2545–2550.
22. Issa, J.-P., Ottaviano, Y., Celano, P., Hamilton, S., Davidson, N. & Baylin, S. (1994) *Nat. Genet.* **7**, 536–540.
23. Herman, J., Latif, F., Weng, Y., Lerman, M. I., Zbar, B., Liu, S., Samid, D., Duan, D. S., Gnarr, J. R., Linehan, W. M., *et al.* (1994) *Proc. Natl. Acad. Sci. USA* **91**, 9700–9704.
24. Herman, J. G., Merlo, A., Mao, L., Lapidus, R. G., Issa, J. P., Davidson, N. E., Sidransky, D. & Baylin, S. B. (1995) *Cancer Res.* **55**, 4525–4530.
25. Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B. & Sidransky, D. (1995) *Nat. Med.* **1**, 686–692.
26. Gonzalez-Zuleta, M., Bender, C., Yang, A., Nguyen, T., Beart, R., Van Tornout, J. & Jones, P. (1995) *Cancer Res.* **55**, 4531–4535.
27. Herman, J. G., Umar, A., Polyak, K., Graff, J., Ahuja, N., Issa, J.-P., Markowitz, S., Willson, J. K. V., Hamilton, S., Kinzler, K., *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95**, 6870–6875.
28. Thibodeau, S., French, A., Cunningham, J., Tester, D., Burgart, L., Roche, P., McDonnell, S., Schaird, D., Vockley, C., Michels, V., *et al.* (1998) *Cancer Res.* **58**, 1713–1718.
29. Koi, M., Umar, A., Chauhan, D. P., Cherian, S. P., Carethers, J. M., Kunkel, T. A. & Boland, C. R. (1994) *Cancer Res.* **54**, 4308–4312.