Commentary

hMLH1 Promoter Hypermethylation in Microsatellite Instability-Positive Endometrial Carcinoma

Cause or Consequence?

Lora Hedrick Ellenson

From the Department of Pathology, Weill Medical College of Cornell University, New York, New York

The prevailing paradigm of cancer as a genetic disorder, arising from the acquisition and subsequent selection of mutations in cancer-causing genes, has propelled much of cancer research over the past two decades and has expanded our understanding of cancer at a phenomenal pace. Cancer-causing genes have been identified at an ever-increasing rate and fall into three major categories: oncogenes, tumor suppressor genes, and the more recently described mutator genes. The development of a malignant neoplasm usually involves mutations in one or more genes of each category, resulting in abnormal function of the encoded protein through changes in the level of gene expression (eg, amplification) or in the characteristic of the protein (eg, missense mutation or translocation). These fundamental changes disturb normal cellular functions (eg, cell cycle control, apoptosis, DNA mismatch repair) that, in sum, drive the neoplastic process. In turn, the perturbation of normal cellular functions has ramifications for the expression status of countless genes, ultimately resulting in the complexity and heterogeneity inherent to malignant neoplasms. The genetic model of cancer would suggest that only those changes in expression caused by a genetic event are critical to the development of the neoplastic process; all others are simply a consequence of the transformed state. However, recent studies of DNA promoter hypermethylation have called this belief into question. Data are accumulating that support a role for this epigenetic alteration in the inactivation of genes that play a causative role in the development of human tumors.

DNA methylation is an epigenetic modification essential to mammalian development.¹ This modification occurs as a postreplicative addition of methyl groups to cytosine residues.² Although this change is reversible by demethylation and, as such, is not a fixed, heritable alteration,

patterns of methylation in normal cells are relatively stable. However, aberrations in DNA methylation including global hypomethylation and localized regions of hypermethylation have been consistently detected in human tumors and transformed cell lines.³⁻⁵ Although the significance of the changes remains controversial, numerous studies support a role for methylation in tumorigenesis. Much of the recent excitement about methylation in cancer has centered around the detection of hypermethylated CpG islands in the promoters of several tumor suppressor genes (eg, p16, von Hippel-Lindau, E-cadherin) and the *hMLH1* mutator gene in tumors that lack intragenic mutations of the respective genes.⁶⁻⁹ The majority of CpG islands in normal cells are unmethylated and the acquisition of methylation at these sites in tumors correlates with loss of gene expression, suggesting that promoter hypermethylation is an alternative mechanism for inactivation of genes that contribute to tumor development.¹⁰ Despite these profound associations, skepticism remains in regard to an active role for methylation in tumorigenesis. This is due in part to our incomplete understanding of the factors that control methylation and the overall consequences of changes in methylation patterns on chromatin structure and gene expression. In this issue of *The American Journal of Pathology*, Esteller and colleagues provide support for an important role of promoter hypermethylation in endometrial tumorigenesis.¹¹ As with most previous studies, theirs does not present direct experimental evidence, but through a series of elegant studies on uterine endometrioid carcinoma (UEC) and its hyperplastic precursor lesions, the authors reveal *hMLH1* promoter hypermethylation as an early event in the development of endometrial carcinoma.

A subset of sporadic uterine endometrioid carcinomas, the most common type of endometrial cancer, demonstrates a molecular phenotype commonly referred to as

Accepted for publication September 20, 1999.

Address reprint requests to Lora Hedrick Ellenson, M.D., Department of Pathology, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021. E-mail: lhellens@mail.med.cornell.edu.

microsatellite instability (MSI).¹²⁻¹⁴ MSI is detected as alterations in the size of microsatellite DNA sequences in DNA derived from tumors compared to DNA from matched normal tissue.¹⁵ This phenotype was first detected in a subset of sporadic colorectal cancer and subsequently shown to be consistently present in tumors from patients with hereditary nonpolyposis colorectal cancer (HNPCC).¹⁶⁻¹⁸ The sequence of events that followed these discoveries was an extraordinary example of the synergy between basic and biomedical science. In brief, soon after MSI was identified in primary human tumors a group working on DNA mismatch repair in *Saccharomyces cerevisiae* reported instability of a dinucleotide repeat (GT) in yeast strains harboring mutations in DNA mismatch repair genes.¹⁹ This provided a connection between phenotype and genotype in a simple eukaryote with possible implications for human tumors. This collision of events expedited the cloning of the first human DNA mismatch repair gene, *hMSH2*, using degenerate primers based on sequences of microbial DNA mismatch repair genes. Furthermore, it was shown that affected HNPCC family members carried germline mutations in *hMSH2* and that the wild-type copy was absent in their colorectal tumors.^{20,21} Soon thereafter, three additional human mismatch repair genes were cloned (*hMLH1*, *hPMS1*, and *hPMS2*) and many HNPCC families were identified with germline mutations in *hMLH1* and only rarely in *hPMS1* and *hPMS2*.^{22,23} Approximately 90% of all HNPCC kindred carry mutations in either *hMLH1* or *hMSH2*. These studies not only defined the cause of an inherited family cancer syndrome, they also provided experimental evidence for what is referred to as the mutator phenotype of cancer and opened a new avenue of cancer research. For an excellent discussion of this topic the reader is referred to a Commentary in the previous volume of the *Journal*.²⁴

Because endometrial carcinoma is the most common non-colorectal cancer in women of HNPCC families, sporadic endometrial carcinomas were tested for MSI and found in a subset of tumors. Notably, the mutational analyses of the four known mismatch repair genes that were shortly to follow were not forthcoming.²⁵⁻²⁸ As the manuscript by Esteller et al notes, of the 61 MSI-positive sporadic UECs analyzed to date only 4 (<7%) contain mutations (3 in *hMSH2* and 1 in *hMLH1*) in one of the four known mismatch repair genes. Studies of sporadic MSI-positive colorectal cancer have produced similar findings.²⁹ These data led investigators to suggest that additional unidentified DNA mismatch repair genes might be responsible for MSI in the majority of sporadic tumors. Recently, however, studies on colorectal, gastric, and endometrial carcinoma have shown a statistically significant correlation between MSI and hypermethylation of the *hMLH1* promoter.³⁰⁻³² For endometrial carcinoma, 71 to 92% of MSI-positive carcinomas have hypermethylation of the *hMLH1* promoter.³²⁻³⁴ Conversely, such methylation is rare in MSI-negative tumors. In addition, several studies using an *in vitro* approach have produced further support for epigenetic silencing of *hMLH1* in the disruption of mismatch repair and subsequent development of microsatellite instability. These investigators have

shown that there are MSI-positive colorectal and endometrial carcinoma cell lines that demonstrate *hMLH1* hypermethylation and lack *hMLH1* expression. When these lines are treated with the demethylating agent 5-azacytidine, *hMLH1* becomes expressed, the MSI phenotype disappears, and functional mismatch repair, as measured by strand-specific mismatch repair, is restored.^{35,36} Furthermore, when the drug is removed, *hMLH1* is silenced and the MSI phenotype is re-established.³⁵ These studies provide strong support for the idea that *hMLH1* promoter hypermethylation is associated with inactivation of a gene with a defined role in the development of human tumors.

However, several aspects of the investigations raise some doubt about a causative role for *hMLH1* hypermethylation in tumor development, including the presence of *hMLH1* promoter hypermethylation in a small number of tumors (sporadic and HNPCC related) with mutations in either *hMLH1* or *hMSH2*; MSI-negative cases with *hMLH1* promoter hypermethylation; and increased tendency of MSI-positive cell lines to methylate endogenous and exogenous DNA.^{37,38} There are several plausible explanations for these findings that do not exclude the importance of *hMLH1* promoter hypermethylation in tumorigenesis.

In the case of associated mutations in *hMLH1*, it has been suggested that promoter hypermethylation may be associated with the silencing of the wild-type allele of *hMLH1*.¹⁰ As with tumor suppressor genes, mutator genes require the inactivation of both alleles to demonstrate the full phenotype: in this circumstance, to abolish mismatch repair activity and promote MSI. The mechanism thought to be most common for inactivation of non-mutated alleles of recessive cancer genes is loss of heterozygosity (LOH) of the region of the chromosome that harbors the gene in question.³⁹ In at least one colorectal cancer with both mutation and hypermethylation of *hMLH1*, loss of heterozygosity of chromosome 3p (the location of *hMLH1*) was lacking, consistent with methylation as an alternative to the inactivation of the wild-type allele.³⁶ Although this argument is convincing, it does not explain the rare occurrence of *hMLH1* promoter methylation in conjunction with *hMSH2* mutation. Investigators have proposed that in this setting *hMLH1* hypermethylation may be monoallelic or heterogeneously present within the tumor cell population.² The current methods used for determination of methylation status are polymerase chain reaction-based and are, therefore, likely to detect low levels of methylation. It has been reported that one such method is capable of detecting 1 methylated allele in 1000 unmethylated alleles.⁴⁰ Given the heterogeneity of malignant neoplasms it is highly likely that in some tumors there is occasional methylation of the CpG island of *hMLH1* without complete loss of *hMLH1* activity and therefore of MSI. This explanation may also explain the occasional MSI-negative tumor that reveals *hMLH1* promoter hypermethylation.

Finally, the propensity of MSI-positive cell lines to methylate DNA fuels the argument that hypermethylation of *hMLH1* may simply reflect an underlying global methylation abnormality. This chicken-and-egg argument is

difficult to combat directly, but data exist that investigators use to support *hMLH1* hypermethylation as the cause and not the consequence of MSI. For example, a paper by Herman et al reports that 22% of HNPCC-related tumors demonstrate p16 hypermethylation.³⁶ This is a much lower incidence than seen in MSI-positive sporadic tumors, where it is 60%, and is comparable to that of MSI-negative sporadic tumors.³⁷ This finding implies that a mismatch repair deficiency *per se* does not necessarily promote hypermethylation of *hMLH1*. Unfortunately, it does not directly address the question of the temporal relationship between *hMLH1* promoter hypermethylation and loss of *hMLH1* expression.

Through the determination of the methylation status of the *hMLH1* promoter in endometrial carcinoma and its hyperplastic precursors, Esteller et al have provided keen insight into this relationship. The authors assayed 27 endometrial carcinomas and 116 endometrial hyperplasias of different severity for *hMLH1* promoter hypermethylation using methylation-specific polymerase chain reaction, an assay developed and used extensively by this group. In addition, in all of the carcinomas and a subset⁴⁰ of the hyperplasias the MSI status was determined. They confirm the association of hypermethylation and MSI in endometrial cancer by showing that 11 of 12 (91%) MSI-positive carcinomas show methylation, whereas all of 15 MSI-negative tumors lack methylation. They go on to show that LOH of chromosome 3p21.3 is absent in all 11 tumors, suggesting that there is biallelic inactivation by *hMLH1* methylation.

Notably, the authors detect methylation in 8 of 116 hyperplasias. Although methylation is absent in 62 simple hyperplasias, it is present in 1 of 33 complex hyperplasias without atypia and in 7 of 21 complex atypical hyperplasias (CAH). CAH is morphologically similar to, but distinguished by lack of stromal invasion from, well differentiated endometrioid carcinoma and is thought to be the direct precursor of the invasive disease. As such, CAH and endometrioid carcinoma are often found concurrently in uteri removed for either diagnosis. Perhaps the most interesting finding of this study is the relationship of hypermethylation, MSI, and the association of hyperplasia and carcinoma.

Of the 8 hypermethylated hyperplasias, 4 displayed MSI and all of these were associated with a concurrent hypermethylated, MSI-positive carcinoma. The 4 remaining cases lacked MSI and only 2 were associated with a carcinoma. Notably, one of the carcinomas was MSI- and methylation-positive and one was MSI-negative and lacked methylation. In the latter case, the authors found an additional focus of CAH that was methylation-negative, suggesting that the invasive component arose from the methylation-negative hyperplasia. Several key points are highlighted by these results. First, the fact that all of the MSI-positive hyperplasias have associated carcinoma is consistent with previous reports. This finding supports a hypothesis first proposed in the adenoma-carcinoma sequence of MSI-positive colorectal carcinoma stating that the temporal window for the development of carcinoma may be relatively short in the presence of MSI. Second, the presence of methylation in

the absence of MSI in two hyperplasias with associated carcinomas suggests that methylation of *hMLH1* can occur before the development of MSI. In one case, the associated carcinoma is MSI- and methylation-positive, suggesting that it arose from the hyperplasia with a methylated *hMLH1* gene in conjunction with the development of MSI. In the other case, the associated carcinoma is both MSI- and methylation-negative, and the authors propose that it arose from the hyperplastic component that lacked methylation. This might be a plausible alternative if the hyperplasia had methylation of only one *hMLH1* allele or the methylation were heterogeneously present. Finally, two of the MSI-negative hyperplasias with methylation lacked associated carcinoma, perhaps reflecting monoallelic/heterogeneous methylation.

In sum, these findings demonstrate that *hMLH1* methylation can precede the development of endometrial carcinoma. However, to determine definitively the consequence of such methylation, additional studies are clearly warranted. To resolve the issue of the relationship of the methylation-positive hyperplasias to the associated carcinomas, the analysis of additional molecular markers altered early in endometrial tumorigenesis (eg, PTEN and K-ras mutations, microsatellites) may be useful. Alternatively, it may be that the definitive answers about the role of promoter hypermethylation in cancer will come unexpectedly from experiments not yet formulated. For today, however, all of the data seem to suggest that promoter hypermethylation is an epigenetic alteration associated with the inactivation of a subset of genes that are central to tumor development.

References

1. Li E, Bestor TH, Jaenisch R: Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 1992, 69:915-926
2. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP: Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* 1998, 72:141-196
3. Goelz SE, Vogelstein B, Hamilton SR, Feinberg AP: Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science* 1985, 228:187-190
4. Feinberg AP, Vogelstein B: Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature* 1983, 301:89-92
5. de Bustros A, Nelkin BD, Silverman A, Ehrlich G, Poiesz B, Baylin SB: The short arm of chromosome 11 is a "hot spot" for hypermethylation in human neoplasia. *Proc Natl Acad Sci USA* 1988, 85:5693-5697
6. Schutte M, Hruban RH, Geradts J, Maynard R, Hilgers W, Rabindran SK, Moskaluk CA, Hahn SA, Schwarte-Waldhoff I, Schmiegel W, Baylin SB, Kern SE, Herman JG: Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. *Cancer Res* 1997, 57:3126-3130
7. Herman JG, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, Samid D, Duan DS, Gnarr JR, Linehan WM, Baylin SB: Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci USA* 1994, 91:9700-9704
8. Graff JR, Herman JG, Lapidus RG, Chopra H, Xu R, Jarrard DF, Isaacs WB, Pitha PM, Davidson NE, Baylin SB: E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res* 1995, 55:5195-5199
9. Cunningham JM, Christensen ER, Tester DJ, Kim CY, Roche PC, Burgart LJ, Thibodeau SN: Hypermethylation of the *hMLH1* promoter in colon cancer with microsatellite instability. *Cancer Res* 1998, 58:3455-3460

10. Jones PA, Laird PW: Cancer epigenetics comes of age. *Nat Genet* 1999, 21:163-167
11. Esteller M, Catus L, Matias-Guiu X, Mutter GL, Prat J, Baylin SB, Herman JG: hMLH1 promoter hypermethylation is an early event in human endometrial tumorigenesis. *Am J Pathol* 1999, 155:1767-1772
12. Risinger JL, Berchuck A, Kohler MF, Watson P, Lynch HT, Boyd J: Genetic instability of microsatellites in endometrial carcinoma. *Cancer Res* 1993, 53:5100-5103
13. Burks RT, Kessis TD, Cho KR, Hedrick L: Microsatellite instability in endometrial carcinoma. *Oncogene* 1994, 9:1163-1166
14. Duggan BD, Felix JC, Muderspach LI, Tourgeman D, Zheng J, Shibata D: Microsatellite instability in sporadic endometrial carcinoma. *J Natl Cancer Inst* 1994, 86:1216-1221
15. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, Meltzer SJ, Rodriguez-Bigas MA, Fodde R, Ranzani GN, Srivastava S: A National Cancer Institute workshop on microsatellite instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. *Cancer Res* 1998, 58:5248-5257
16. Thibodeau SN, Bren G, Schaid D: Microsatellite instability in cancer of the proximal colon. *Science* 1993, 260:816-819
17. Ionov Y, Peinado MA, Malkhosyan S, Shibata D, Perucho M: Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 1993, 363:558-561
18. Aaltonen LA, Peltomäki P, Leach FS, Sistonen P, Pylkkänen L, Mecklin JP, Powell SM, Jen J, Hamilton SR, Petersen GM, Kinzler KW, Vogelstein B, de la Chapelle A: Clues to the pathogenesis of familial colorectal cancer. *Science* 1993, 260:812-816
19. Strand M, Prolla TA, Liskay RM, Petes TD: Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. *Nature* 1993, 365:274-276
20. Fishel R, Lescoe MK, Rao MR, Copeland NG, Jenkins NA, Garber J, Kane M, Kolodner R: The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell* 1993, 75:1027-1038
21. Liu B, Parsons RE, Hamilton SR, Petersen GM, Lynch HT, Watson P, Markowitz S, Willson JKV, Green J, de la Chapelle A, Kinzler KW, Vogelstein B: hMSH2 mutations in hereditary nonpolyposis colorectal cancer kindreds. *Cancer Research* 1994, 54:4590-4594
22. Papadopoulos N, Nicolaides NC, Wei YF, Ruben SM, Carter KC, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, Adams MD, Venter JL, Hamilton SR, Petersen GM, Watson P, Lynel HT, Peltomäki P, Jakka-Pekka Mecklin, de la Chapelle A, Kinzler KW, Vogelstein B: Mutation of a mutL homolog in hereditary colon cancer. *Science* 1994, 263:1625-1629
23. Nicolaides NC, Papadopoulos N, Liu B, Wei YF, Carter KC, Ruben SM, Rosen CA, Haseltine WA, Fleischmann RD, Fraser CM, Adams MD, Venter JC, Dunlop MG, Hamilton SR, Petersen GM, de la Chapelle A, Vogelstein B, Kinzler KW: Mutations of two PMS homologues in hereditary nonpolyposis colon cancer. *Nature* 1994, 371:75-80
24. Loeb KR, Loeb LA: Genetic instability and the mutator phenotype: studies in ulcerative colitis. *Am J Pathol* 1999, 154:1621-1626
25. Kowalski LD, Mutch DG, Herzog TJ, Rader JS, Goodfellow PJ: Mutational analysis of MLH1 and MSH2 in 25 prospectively-acquired RER endometrial cancers. *Genes Chromosomes Cancer* 1997, 18:219-227
26. Katabuchi H, van Rees B, Lambers AR, Ronnett BM, Blazes MS, Leach FS, Cho KR, Hedrick L: Mutations in DNA mismatch repair genes are not responsible for microsatellite instability in most sporadic endometrial carcinomas. *Cancer Res* 1995, 55:5556-5560
27. Lim PC, Tester D, Cliby W, Ziesmer SC, Roche PC, Hartmann L, Thibodeau SN, Podratz KC, Jenkins RB: Absence of mutations in DNA mismatch repair genes in sporadic endometrial tumors with microsatellite instability. *Clin Cancer Res* 1996, 2:1907-1911
28. Kobayashi K, Matsushima M, Koi S, Saito H, Sagae S, Kudo R, Nakamura Y: Mutational analysis of mismatch repair genes, hMLH1 and hMSH2, in sporadic endometrial carcinomas with microsatellite instability. *Jpn J Cancer Res* 1996, 87:141-145
29. Moslein G, Tester DJ, Lindor NM, Honchel R, Cunningham JM, French AJ, Halling KC, Schwab M, Goretzki P, Thibodeau SN: Microsatellite instability and mutation analysis of hMSH2 and hMLH1 in patients with sporadic, familial and hereditary colorectal cancer. *Hum Mol Genet* 1996, 5:1245-1252
30. Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, Jessup JM, Kolodner R: Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Research* 1997, 57:808-811
31. Fleisher AS, Esteller M, Wang S, Tamura G, Suzuki H, Yin J, Zou TT, Abraham JM, Kong D, Smolinski KN, Shi YQ, Rhyu MG, Powell SM, James SP, Wilson KT, Herman JG, Meltzer SJ: Hypermethylation of the hMLH1 gene promoter in human gastric cancers with microsatellite instability. *Cancer Res* 1999, 59:1090-1095
32. Esteller M, Levine R, Baylin SB, Ellenson LH, Herman JG: MLH1 promoter hypermethylation is associated with the microsatellite instability phenotype in sporadic endometrial carcinomas. *Oncogene* 1998, 17:2413-2417
33. Simpkins SB, Bocker T, Swisher EM, Mutch DG, Gersell DJ, Kovatich AJ, Palazzo JP, Fishel R, Goodfellow PJ: MLH1 promoter methylation, and gene silencing is the primary cause of microsatellite instability in sporadic endometrial cancers. *Hum Mol Genet* 1999, 8:661-666
34. Gurin CC, Federici MG, Kang L, Boyd J: Causes and consequences of microsatellite instability in endometrial carcinoma. *Cancer Res* 1999, 59:462-466
35. Veigl ML, Kasturi L, Olechnowicz J, Ma AH, Lutterbaugh JD, Periyasamy S, Li GM, Drummond J, Modrich PL, Sedwick WD, Markowitz SD: Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. *Proc Natl Acad Sci USA* 1998, 95:8698-8702
36. Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, Markowitz S, Willson JK, Hamilton SR, Kinzler KW, Kane MF, Kolodner RD, Vogelstein B, Kunkel TA, Baylin SB: Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci USA* 1998, 95:6870-6875
37. Ahuja N, Mohan AL, Li Q, Stolker JM, Herman JG, Hamilton SR, Baylin SB, Issa JP: Association between CpG island methylation and microsatellite instability in colorectal cancer. *Cancer Res* 1997, 57:3370-3374
38. Lengauer C, Kinzler KW, Vogelstein B: DNA methylation and genetic instability in colorectal cancer cells. *Proc Natl Acad Sci USA* 1997, 94:2545-2550
39. Vogelstein B, Fearon ER, Kern SE, Hamilton SR, Preisinger AC, Nakamura Y, White R: Allelotype of colorectal carcinomas. *Science* 1989, 244:207-211
40. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB: Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996, 93:9821-9826