Inheritance of a Cancer-Associated \textit{MLH1} Germ-Line Epimutation

Megan P. Hitchins, Ph.D., Justin J.L. Wong, B.Sc., Graeme Suthers, M.B., B.S., Catherine M. Suter, Ph.D., David I.K. Martin, M.D., Nicholas J. Hawkins, Ph.D., and Robyn L. Ward, Ph.D.

Persons who have hypermethylation of one allele of \textit{MLH1} in somatic cells throughout the body (a germ-line epimutation) have a predisposition for the development of cancer in a pattern typical of hereditary nonpolyposis colorectal cancer. By studying the families of two such persons, we found evidence that the epimutation was transmitted from a mother to her son but was erased in his spermatozoa. The affected maternal allele was inherited by three other siblings from these two families, but in those offspring the allele had reverted to the normal active state. These findings demonstrate a novel pattern of inheritance of cancer susceptibility and are consistent with transgenerational epigenetic inheritance.

\textbf{SUMMARY}

Persons who have hypermethylation of one allele of \textit{MLH1} in somatic cells throughout the body (a germ-line epimutation) have a predisposition for the development of cancer in a pattern typical of hereditary nonpolyposis colorectal cancer. By studying the families of two such persons, we found evidence that the epimutation was transmitted from a mother to her son but was erased in his spermatozoa. The affected maternal allele was inherited by three other siblings from these two families, but in those offspring the allele had reverted to the normal active state. These findings demonstrate a novel pattern of inheritance of cancer susceptibility and are consistent with transgenerational epigenetic inheritance.
This process removes and resets epigenetic marks between generations. Nevertheless, nonmendelian patterns of transgenerational epigenetic inheritance have been reported in mice. In the eight previously reported cases of germ-line MLH1 epimutation in humans, no intergenerational transmission was found, although in one patient the epimutation was reported in a low proportion of spermatozoa.

Since the presence of an MLH1 epimutation in the preimplantation embryo removes and resets epigenetic marks between generations. Nevertheless, nonmendelian patterns of transgenerational epigenetic inheritance have been reported in mice. In the eight previously reported cases of germ-line MLH1 epimutation in humans, no intergenerational transmission was found, although in one patient the epimutation was reported in a low proportion of spermatozoa.

Since the presence of an MLH1 epimutation in the preimplantation embryo removes and resets epigenetic marks between generations. Nevertheless, nonmendelian patterns of transgenerational epigenetic inheritance have been reported in mice. In the eight previously reported cases of germ-line MLH1 epimutation in humans, no intergenerational transmission was found, although in one patient the epimutation was reported in a low proportion of spermatozoa.

Since the presence of an MLH1 epimutation in the preimplantation embryo removes and resets epigenetic marks between generations. Nevertheless, nonmendelian patterns of transgenerational epigenetic inheritance have been reported in mice. In the eight previously reported cases of germ-line MLH1 epimutation in humans, no intergenerational transmission was found, although in one patient the epimutation was reported in a low proportion of spermatozoa.
Panel A shows a map of SNPs within the MLH1 and EPM2AIP genes, used to determine inheritance patterns of the epimutant alleles and to analyze allelic expression. Panels B and C show pedigrees for Patient A and Patient B, with large black circles denoting Patient A and Patient B and with the current age of each family member given. Generations are listed I to III, and patients are identified by number. In Panels B and C, combined bisulfite restriction analysis of the C region of MLH1 (digested with MluI) is shown, with the lanes corresponding to data for family members shown in the pedigrees directly above. Combined bisulfite restriction analysis was performed on DNA extracted from peripheral-blood leukocytes from all patients except family member II7–A and family member II8–A, from whom only hair-follicle DNA was available. Lane M indicates the pUC19/MspI DNA ladder, lane C– indicates DNA extracted from peripheral-blood leukocytes from a control with an unmethylated MLH1 promoter, and lane C+ indicates DNA extracted from the biallelically methylated RKO colorectal-carcinoma cell line (ATCC). Band sizes (in base pairs) are shown at the right. At the bottom of Panels B and C, haplotypes generated from informative SNPs are listed according to the key for each pedigree. Alleles associated with the epimutation are highlighted in yellow, and the presence of methylation (Me) is indicated. Maternally inherited alleles are shown in red, and paternally derived alleles are shown in blue; black letters indicate unknown parental origin. Although the haplotypes associated with the epimutation were inherited by several children, only family member I16–A retained the epimutation.

**Methods**

**Patients and Family Members**

Our study was approved by the ethics committee at St. Vincent’s Hospital in Sydney. Tissues were obtained with written informed consent from probands and family members at St. Vincent’s Hospital and Women’s and Children’s Hospital in Adelaide, Australia.

The New England Journal of Medicine
Downloaded from nejm.org at UQ Library on March 26, 2017. For personal use only. No other uses without permission. 
Copyright © 2007 Massachusetts Medical Society. All rights reserved.
We selected 24 patients in whom colorectal or endometrial cancer had developed before the age of 50 years and who lacked deleterious germ-line sequence mutations in MSH2 or MLH1. In each case, the tumors had microsatellite instability, with complete loss of MLH1 protein expression and retention of MSH2. Additional tissues were collected from patients shown to carry an MLH1 germ-line epimutation and from their first-degree relatives. The “swim-up” procedure was used to isolate motile spermatozoa.\textsuperscript{15} To remove contaminating somatic cells, spermatozoa that were used for DNA analyses were additionally sorted by flow cytometry (FACSVantage DeVa, Becton Dickinson).\textsuperscript{16} DNA was extracted from spermatozoa, hair follicles, and buccal mucosa with the use of QuickExtract solution (Epicentre Biotechnologies) and from peripheral-blood leukocytes with the use of phenol–chloroform.

**Methylation Analyses**

Methylation of the promoter of MLH1 (A and C regions) and of the EPM2AIP promoter on the opposite strand was identified with the use of combined bisulfite restriction analyses (Fig. 1A) and confirmed by allelic bisulfite sequencing.\textsuperscript{7}

Quantitative real-time methylation-specific polymerase-chain-reaction (PCR) assays were performed on bisulfite-treated DNA with the use of primers specific to methylated templates for the C region of MLH1 and the imprinted SNRPN gene.\textsuperscript{17} Primers that amplify the control gene MyoD regardless of its methylation status were used to normalize for DNA input. Real-time methylation-specific PCR assays with iQ SYBR Green Supermix reagent were analyzed with the use of a real-time PCR system (MyiQ, BioRad). Absolute values for experimental samples were calculated from the PCR cycle number at which the fluorescence crossed the threshold with the use of a standard curve. The percentage of methylated alleles in the C region of MLH1 and SNRPN was calculated against MyoD with reference to 100% in vitro methylated human DNA (Chemicon).\textsuperscript{18} All primer sequences are available on request.

**Haplotyping**

Single-nucleotide polymorphism (SNP) typing was performed by PCR amplification of constitutional DNA followed by restriction digestion or direct sequencing of the purified amplicons. Markers of sequence-tagged sites were typed by PCR amplification with the use of fluorescent-labeled primers,\textsuperscript{19} separated by capillary electrophoresis on an automated DNA sequencer, and sized with the use of LIZ markers (ABI 3700, Applied Biosystems).

**Allelic Expression**

For each patient or family member, heterozygous polymorphisms within exons of MLH1 and EPM2AIP were used to identify the alleles being transcribed.\textsuperscript{20} RNA was extracted from peripheral-blood leukocytes, lymphoblastoid cells, and...
spermatozoa with the use of Trizol reagent (Invitrogen), treated with DNaseI, and converted to complementary DNA (cDNA). Allelic expression was determined by sequencing cDNA at I219V SNP within exon 8 (rs1799977) and at polymorphisms within exon 16 and EPM2AIP.

### RESULTS

By studying the peripheral blood of 24 patients, we identified two unrelated women (Patient A and Patient B) who had the typical molecular and clinical characteristics of persons with germ-line MLH1 epimutations — namely, multiple MLH1-negative cancers of the colorectum and endometrium and hemiallelic methylation of MLH1 in all somatic cells. In both women, there was dense methylation of one allele of the MLH1 and EPM2AIP promoters in somatic cells from the three embryonic germ layers (Fig. 1B, and Fig. 1A of the Supplementary Appendix, available with the full text of this article at www.nejm.org). Both women had metachronous carcinomas that had microsatellite instability and lacked MLH1 expression. (Patient A received a diagnosis of cancer of the endometrium at the age of 45 years, of the colon at 59 years,
and of the rectum at 60 years; Patient B received a diagnosis of cancer of the colon at 41 years and of the rectum at 45 years.) Patient A was heterozygous for a SNP (rs1800734) within the MLH1 promoter, with methylation confined to the A allele. In both Patient A and Patient B, the methylated allele was transcriptionally silent, as evidenced by monoallelic expression of MLH1 and EPM2AIP transcripts in their messenger RNA (mRNA) (Fig. 1C and 1D, and Fig. 1B of the Supplementary Appendix).

To identify MLH1 epimutations within families of the probands, combined bisulfite restriction analysis was performed on constitutional DNA from nine first-degree relatives, none of whom had a history of cancer (Fig. 2B and 2C). Partial methylation of MLH1 was found in one of Patient A’s four sons (family member II6-A) (Fig. 2C). Methylation of the A allele (SNP rs1800734) on approximately 50% of chromosomes was confirmed by bisulfite sequencing (Fig. 3A). We identified an expressible C→T SNP within MLH1 exon 16 in family member II6-A, which was used to demonstrate that he was transcribing RNA only from the MLH1 allele inherited from his father (Fig. 3A). These data are consistent with transmission of the MLH1 epimutation from Patient A to her son.

To ascertain the possibility of transmission of the MLH1 epimutation from family member II6-A to his offspring, we studied the level of allelic methylation in his pure motile spermatozoa with the use of a sensitive quantitative real-time methylation assay within the C region of MLH1 (Fig. 1A). In DNA from peripheral-blood leukocytes obtained from family member II6-A, approximately half of the MLH1 alleles (mean ±SD, 42.0±4.6%) were methylated. In contrast, his sperm had no trace of MLH1 methylation, suggesting in trans or subsequently somatic methylation. With the use of real-time methylation-specific PCR, we found no evidence of allelic mosaicism for MLH1 methylation in any patient in our study, except those with epimutations (38±9% for Patient B, 42±7% for Patient A, and 42±4.6% for family member II6-A).

Epimutations are meiotically reversible and often show somatic mosaicism. We therefore considered whether some family members had low levels of allelic MLH1 methylation, suggesting incomplete somatic erasure of an epimutation, or a susceptibility of the allele to subsequent somatic methylation. With the use of real-time methylation-specific PCR, we found no evidence of allelic mosaicism for MLH1 methylation in any patient in our study, except those with epimutations (38±9% for Patient B, 42±7% for Patient A, and 42±4.6% for family member II6-A).

**DISCUSSION**

We have found evidence of germ-line epimutation of MLH1 in a woman with cancer and in her son (Fig. 4), which supports the concept of trans-
Figure 4. Schematic Representation of Inheritance of MLH1 Alleles in Three Sons of Patient A.
The diagram depicts MLH1 alleles within the nucleus of somatic cells and sperm, with pink representing maternal origin and blue representing paternal origin. Active transcription is indicated by an arrow, and the RNA transcripts from the alleles are shown within cytoplasm. In the mother, one allele was methylated in the promoter (red band) and therefore not transcribed; only RNA from her yellow allele was expressed. In her second son (family member II6-A), the maternally inherited allele (pink, red band) was silent, and the paternal allele (pale blue) was expressed. Haploid DNA within spermatozoa from the son contained alleles from both the father and mother, and both were transcribed. There was no evidence of methylation of either allele. The first and third sons (family members II5-A and II7-A) also inherited the affected maternal allele, but in these offspring methylation had been erased and biallelic expression of MLH1 was found, indicating that the epimutation was reversed to the normal state. This model shows transgenerational inheritance of an epigenetically mutated tumor-suppressor gene and subsequent reversal of the epimutation within spermatozoa.
generational epigenetic inheritance. The MLH1 epimutation that predisposed the mother to multiple tumors with microsatellite instability has increased the risk of cancer in her son (family member II6-A).

The findings from the two families in this study, as well as previous studies, offer insights into the pattern of inheritance of germ-line epimutations. With reference to the parent of origin, the mother of family member II6-A, like the mothers of two patients with MLH1 epimutations reported previously, had cancer in a pattern typical of hereditary nonpolyposis colorectal cancer. Furthermore, in two other cases in which germ-line epimutations were shown to arise spontaneously (Patient B and a patient whose case was reported previously), the methylated allele was maternally derived. Taken together, these data raise the possibility that epigenetic errors may arise more frequently during oogenesis or are more likely to be maintained during this process. Our finding of erasure of the epimutation during spermatogenesis in family member II6-A is consistent with this hypothesis. However, paternal inheritance cannot be excluded, given our previous finding of low-level MLH1 methylation in sperm of an affected person.

Another characteristic of the inheritance pattern in the families in this study is that four sons inherited their mother’s MLH1 haplotype, yet in three of the sons, the maternally derived allele had undergone demethylation and transcriptional reactivation. It appears that the normal process of gametogenesis allowed correction of the MLH1 epimutation, perhaps contemporaneously with erasure of parent-specific methylation of imprinted genes in primordial germ cells. If so, then transmission of an epimutation to family member II6-A must reflect resistance to reprogramming, either through incomplete erasure or by retention of an epigenetic memory. Although at present we have limited information, the overall cancer risk for families with germ-line MLH1 epimutations appears to be lower than for those with germ-line sequence mutations.

An alternative explanation for our findings is that epimutations are not inherited per se. Rather, they are erased in gametogenesis but reestablished in successive generations because of cis-acting or even trans-acting genetic factors that increase susceptibility to MLH1 epimutations. Examples of epigenetic silencing that are driven by genetic events in cis include deletion of imprint-control centers in imprint disorders and expansion of triplet repeats within the FMR1 promoter in the fragile X syndrome. Such a mechanism may also explain the recently reported strongly heritable pattern of epimutation in MSH2 since the methylation state segregated faithfully with the genetic haplotype. In contrast, in the two families described in our study, we found no evidence of a fully penetrant in cis defect. Rather, they showed epimutations that were meiotically reversible and transmitted in a nonmendelian fashion. A simple explanation for this pattern is that epimutations can occur on any haplotype, and although they usually are cleared in the germ line, they may be retained at low but uncertain frequency.

Regardless of uncertainties about patterns, frequencies, and mechanisms of inheritance, offspring of patients with MLH1 epimutations must be regarded not as being at risk for cancer until proven otherwise. The broader implication of this study is that disease states in humans may be the consequence of nonmendelian inheritance of epigenetic changes in one or more genes.

Supported by the National Health and Medical Research Council, the Cancer Council New South Wales, and the Justin O’Connor Foundation. Mr. Wong is the recipient of a University of New South Wales International Postgraduate Research Scholarship. No potential conflict of interest relevant to this article was reported.

We thank Kay Fong Cheong, Deborah Packham, Vita Ap Lin, and Chau-To Kwok for their technical assistance and Rachel Williams and Kerry Phillips for their coordination of sample collection.

REFERENCES
6. Suter CM, Martin DI, Ward RL. Germ-