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Clustering of Lynch syndrome malignancies with no evidence for a role of DNA Mismatch Repair

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Abstract

Objectives—We ascertained a large kindred with an excess of Lynch syndrome-associated cancers. Our objective was to determine if a defect in one of the DNA mismatch repair (DMMR) genes was the probable cause of cancer susceptibility as microsatellite instability (MSI) and immunohistochemical (IHC) analysis of the probands' tumors did not provide a clear indication.

Methods—A detailed history and review of medical records was undertaken to construct a four-generation pedigree. Blood samples were obtained for analysis of germline DNA. Polymorphic repeats from the MLH1, MSH2, MSH6, and PMS2 loci were genotyped and the co-segregation of markers and disease was assessed. DMMR gene expression for all available tumors was evaluated by IHC. Combined bisulfite restriction analysis (COBRA) of MLH1 was utilized to test for germline epimutation.

Results—Four gynecologic carcinomas, 3 colon carcinomas, and 13 cases of adenomatous polyps were identified. The family met Amsterdam II criteria. The mean age of cancer diagnosis in the kindred was 63 years (range 44–82). DNA marker analyses excluded linkage to MLH1, MSH2, MSH6, and PMS2. Furthermore, MSI and IHC analysis of tumors did not suggest a role for DMMR. Methylation of the MLH1 promoter was identified in the peripheral blood leukocytes (PBLs) of a family member with an early onset colon cancer.

Conclusions—We identified a large family with multiple Lynch malignancies and no evidence for an inherited defect in DMMR. This family represents an important but poorly understood form of autosomal dominant inherited cancer susceptibility. Aberrant MLH1 promoter methylation in normal tissues may be a marker for cancer susceptibility in families such as this.

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Introduction

Lynch syndrome, a subset of Hereditary Non-Polyposis Colorectal Cancer (HNPCC), is an autosomal dominant cancer susceptibility syndrome characterized by inherited defects in the DNA mismatch repair system (DMMR) (1). Family members with this syndrome have an increased risk of colorectal (CRC), endometrial, ovarian, stomach, kidney, ureter, small intestine and hepatobiliary tract malignancies (2). Penetrance is approximately 80% in classic Lynch syndrome families, synchronous or metachronous cancers are common, diagnoses are typically at a younger age, and colorectal cancers are more commonly right-sided with poorly differentiated histology (2).

Amsterdam criteria were created as a screening tool, initially focusing on CRC susceptibility, to provide a common nomenclature for the selection of families with a high likelihood of having HNPCC. At that time, the genes and their functions had not been identified and characterized, so the diagnosis was based on the family history and the age of onset of colorectal cancer. Original Amsterdam I criteria included three family members with colorectal cancer, one being a first-degree relative of the other two, at least two generations affected, and at least one cancer diagnosed before age 50 (3). These criteria have been expanded to include extra-colonic malignancies and other risk factors for genetic disease (Amsterdam II and Bethesda criteria) (4,5). The term Lynch syndrome has been suggested as more appropriate than HNPCC as this name clarifies the consideration of non-colonic cancers in a family history and unifies the diagnosis around the germline mutation in a DNA mismatch repair gene (6). It is well known that not all HNPCC defined families (Amsterdam-positive criteria) have true Lynch syndrome and in many cases MSI of tumors exist without vertical transmission of an aberrant DNA mismatch repair gene. The term Lynch syndrome emphasizes the hereditary predisposition of malignancy that is due to germline mutation in one of the DMMR genes. Extra-colonic malignancies are especially prevalent in women with Lynch syndrome as they have a higher risk of endometrial cancer than CRC (7). The identification of Lynch syndrome families is of paramount importance as these individuals may benefit from genetic counseling, genetic testing, and more intense cancer surveillance (8).

Germline mutations in the DMMR genes MLH1 and MSH2 are responsible for the majority of Lynch syndrome families; however, mutations in MSH6 and PMS2 also lead to a Lynch syndrome phenotype (9). The loss of DMMR leads to genomic instability by increasing the frequency of sporadic mutations in both coding and non-coding sequences.

Studies of families with Lynch syndrome have defined the role of DMMR genes in the molecular pathogenesis of Lynch syndrome associated malignancies. We identified a large family with an excess number of Lynch syndrome associated malignancies over three generations including colorectal, endometrial and ovarian cancers. Amsterdam II criteria were met as several members of this family had early onset-disease and cancer penetrance was high. We sought to determine if DMMR was responsible for the genetic cancer susceptibility in this family. Whereas it has been suggested that mismatch repair gene mutations are the underlying cause of HNPCC, an undetermined and likely underestimated number of families seem to lack these defects.

Methods

Clinical Data

This study was approved by the Human Research Protection Office at Washington University and patient consent was obtained for release of all medical records and tissue specimens. A detailed family history was obtained through several family interviews and a

thorough review of the medical records. Peripheral blood was collected from 38 family members as a source of germline DNA. Additional tissue samples including hair, buccal cells, and urine were collected from four affected family members (2 colon cancer, 1 endometrial cancer, and 1 endometrial hyperplasia and colon adenoma) to serve as sources of germline DNA. Formalin-fixed, paraffin embedded tumor tissues were obtained when available for all Lynch syndrome-associated tumors.

DNA Preparation

Normal DNA was isolated from peripheral blood lymphocytes (PBLs) using the Qiagen DNA blood Midi kit (Qiagen, Valencia, CA). All blood was drawn at the study institution or an outside hospital and shipped overnight to the study institution where it was labeled and frozen upon retrieval in the laboratory. Genomic DNA from tumor tissues, hair shaft roots, buccal cells, and bladder epithelial cells was extracted with Qiagen DNeasy Tissue kit (Qiagen, Valencia, CA).

Microsatellite Instability

Microsatellite instability was assessed using PCR based assays as previously described (10,11). Tumor and corresponding normal DNA was genotyped using the five National Cancer Institute consensus microsatellite markers (D5S346, D2S123, D17S250, BAT-25, and BAT-26). Tumors were designated as having high-level MSI (MSI-H) if novel PCR bands were present in a least two of the five markers, low-level MSI (MSI-L) if a single marker demonstrated MSI or microsatellite stable (MSS) if there was no evidence of MSI in any marker.

Immunohistochemistry for DNA MMR proteins

Immunohistochemistry for the DMMR proteins, MLH1, MSH2, MSH6, and PMS2 was performed using 5 µm-thick tissue sections from formalin-fixed, paraffin-embedded tissue as previously described (12). Tissues were stained with antibodies to hMLH1 (clone G16H 728; 1 mg/ml [PharMingen]), hMSH2 (clone FE11; 0.5 mg/ml [Oncogene Science]), hMSH6 (clone 44, 0.5 µg/ml [Transduction Laboratories]), and hPMS2 (clone A16-4, BD Pharmingen). Tumor cells that showed an absence of nuclear staining in the presence of normal positive staining in surrounding cells were interpreted as negative for expression of the respective proteins.

DNA Marker Linkage Studies

Polymorphic markers including microsatellite repeats and single nucleotide polymorphism (SNPs) were identified within the genomic sequence or in the flanking regions of the MLH1, MSH2, MSH6 and PMS2 DMMR genes. Three highly informative microsatellites from the MSH2-MSH6 region on chromosome 2p (47,483,767-47,887,588) were evaluated. An ATnACAGn complex repeat approximately 155 kb from the 3' end of MSH2 (primers: F 5'-GAAGCTTAAGCCAAAAGGGTGA-3'; R 5'-GCCAATCCCTCCTGGTAAAT-3'), D2S123 (primers: F 5'-AAACAGGATGCCTGCCTTTA-3'; R 5'-GGACTTTCCACCTATGGGAC-3'), and a CAn repeat approximately 97 kb 3' of MSH6 (primers: F 5'-TGGTGGTTACCAGACAGTGC-3'; R 5'-CGTCTCCTAGTGGGGTTTGA-3') were genotyped using PCR methods. Alleles were detected using γ -³²P ATP-labeling, size sorting on a 6% denaturing acrylamide gel and autoradiography. Individual marker genotypes and haplotypes for the 2p region were determined. Two MLH1 markers were typed: a TAnTn complex repeat in intron 11 (primers: F 5'-CCATTGGGGACCTGTATATC-3'; R 5'-CGAGGTCAGACTTGTGTGG-3') and the SNP (rs1800734) located 93 bp 5' of the MLH1 initiation codon (primers F 5'-CGCAAGCGCATATCCTTCTA-3' R 5'-CGTCTAGATGCTCAACGGAAG-3'). The -93

SNP gives rise to an A/G nucleotide polymorphism that is utilized for genotyping with PvuII restriction digest as previously described (13). A single PMS2 GTn microsatellite marker (primers F 5'- CCCCCACTGTCTCCAAAAA -3'; R 5'- TCTCATTTCTCACCCCCATATT-3') and one SNP (rs1805321) were investigated. The PMS2 SNP gives rise to an A/G polymorphism that lends itself to genotyping with MboI restriction digest (primers F 5'- AGCCTCACAGCCCCAAAGACT-3'; R 5'- GGAGCTGGCCGCATACTC-3'). PCR conditions are depicted in supplementary table 1.

Methylation Analysis

Methylation was assessed using combined bisulfite restriction analysis (COBRA). DNA bisulfite conversion was performed using a commercially available kit (EZ DNA Methylation Gold Kit, Zymo Research, Orange CA). The proximal region of the MLH1 promoter (-333 to -218) was assessed as previously described (14). Methylation of this region has been previously shown to correlate with lack of MLH1 expression and thus been defined as the "critical region" within the MLH1 promoter (15). Two additional MLH1 COBRA assays (-751 to -573 and -26 to +113) were evaluated along with sequences corresponding to the MINT1,-2,-12,-25, and -31 loci (16,17). PCR primers, product sizes, and restriction sites evaluated are presented in supplemental Table 1. Unrestricted and restricted PCR products were resolved on 10% nondenaturing polyacrylamide gels, stained with ethidium bromide and photoimaged with a UV camera (ImageStore 500 Version 7.12, White/UV Transilluminator; UVP, Inc., Upland, CA). Methylation was present if specific methylation restriction fragments were demonstrated.

Bisulfite converted PCR products from MLH1 COBRA demonstrating methylation in the promoter were purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and cloned into E coli using the TOPO TA cloning system (Invitrogen, Carlsbad, CA). Following transformation, vector-specific primers were used to verify expected size inserts. Bacterial clones were grown overnight and plasmid DNA was prepared using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). 42 clones underwent direct sequencing using ABI Prism BigDye Terminator chemistry v1.1 (Applied Biosystems, Foster City, CA).

Results

Pedigree Analysis

The four-generation cancer history strongly suggested autosomal dominant cancer susceptibility (Figure 1). The family fulfilled Amsterdam II criteria for Lynch syndrome (HNPCC). Confirmed Lynch syndrome-associated malignancies included three colon (individuals II-1, II-6, III-4), three endometrial (individuals I-2, II-3, II-5), and one ovarian (individual II-8). In addition to these cancers, 13 adenomatous colon polyps (all confirmed by review of medical records) were seen in four family members (individuals II-5, II-7, III-2, III-4). Tumor characteristics are described in Table 1. Four additional family members had confirmed non-Lynch syndrome-associated malignancies including prostate (2), breast (1), and squamous cell cancer of the neck (1) (Figure 1). Two cancers (one colon and one ovarian) were diagnosed prior to age 50; however, the median age at diagnosis of Lynch syndrome-associated tumors was 64 years (range 44-82). All three colorectal cancers were left-sided.

Tumor Studies

Six Lynch syndrome-associated tumors from five affected family members were evaluated for defects in DMMR by IHC and/or MSI based on tumor availability (Table 1). Of those analyzed, only one, an endometrial cancer (proband II-5), had a demonstrable defect in DMMR. The endometrial cancer was MSI-H and IHC revealed loss of MLH1 expression

(Figure 2). COBRA showed methylation of the MLH1 promoter explaining gene silencing and loss of MLH1 in this tumor (data not shown).

DNA Marker Linkage Studies

DNA marker genotyping studies for 39 family members (indicated with asterisks in Figure 1) served to exclude linkage between MLH1, MSH2, MSH6, and PMS2 and the cancer susceptibility locus. Linkage studies were particularly important in this family as it was unclear which if any of the DMMR genes were involved based on initial MSI and IHC data. Figure 2 illustrates haplotypes for the MSH2-MSH6 region of chromosome 2p in family members with confirmed Lynch syndrome-associated cancers or pre-cancers. Linkage between MSH2 and MSH6 and cancer susceptibility was excluded as affected individuals did not share the same haplotype. Although the 133 haplotype was shared by affected individuals in the right branch of the pedigree (I-2, II-5, II-6, II-7), none of those on the left had this same haplotype. The four affected individuals in the left branch of the pedigree did not share the same haplotype. Linkage to MLH1 and PMS2 was similarly excluded (data not shown).

MLH1 Methylation in Normal Tissues

Germline MLH1 epimutation was investigated by COBRA of normal DNA (PBLs) from 6 family members with documented cancers or pre-cancers (individuals II-1, II-3, II-5, II-7, III-2, and III-4). Investigation of PBLs allowed for investigation of a epimutation in the germline as opposed to a mutation that developed in the tumor alone. Methylation of the proximal region of the MLH1 promoter (-333 to -218) has been previously shown to correlate with lack of MLH1 expression and thus been defined as the “critical region” within the promoter (15). This area of the promoter is illustrated in Figure 3A as assay number two. Methylation at CpG sites outside of the critical region was also assessed by COBRA (Figure 3A, Assays number 1 and 3). Methylation at positions -250, -252, and -262 within assay 2 was seen in the normal blood DNA of one family member with an early-onset colon cancer (individual III-4) (Figure 3B). The 5' and 3' sequences investigated (assays number one and three) showed no methylation of CpG sites in other areas of the promoter for this individual. Methylation of the “critical region” within the MLH1 promoter was confirmed with repeat DNA extraction and bisulfite conversion. Bisulfite cloning and sequencing demonstrated dense methylation with 16% of clones (6 of 37) methylated for all 8 cytosine residues within assay 2 (Figure 3C). No methylation was seen in other tissue specimens (hair, buccal cells, bladder epithelium) from either individual III-4 nor three other family members (individuals II-1, II-3, and III-2).

The CpG island methylator phenotype (CIMP) has been thoroughly described in colorectal cancer and to a lesser extent in other solid tumors including endometrial cancer (16,17,18). This phenotype is often associated with distinctive clinical characteristics and prognosis. We investigated markers of CIMP previously described (MINT1, MINT2, MINT12, MINT25, and MINT 31) as this has been proposed as an objective approach to defining a methylator phenotype. No methylation in PBLs was discovered in MINT markers among 6 family individuals with cancer or pre-cancer who were also studied for MLH1 methylation (individuals II-1, II-3, II-5, II-7, III-2, and III-4) (data not shown).

Discussion

Families with a diagnosis of Lynch syndrome have been shown to carry germline mutations in one of the DMMR genes with linkage analysis mapping the majority of these defects to MLH1, MSH2 and MSH6 (19). The family we describe fulfills the clinical diagnosis of Lynch syndrome (Amsterdam II criteria), yet we have excluded linkage between MLH1,

MSH2, MSH6 and PMS2 and the cancer susceptibility locus through genotype and haplotype analysis. By the Lynch definition of defects in DMMR genes, this family cannot be termed true Lynch syndrome due to the lack of mutation in a DMMR gene. Families such as this one without a defined mutator phenotype have been described previously and represent an important group for molecular and clinical study (20-22). Mutations in the DMMR genes are only identified in 50-70% of families meeting stringent Amsterdam I criteria (20). Those families that fit Amsterdam I or II criteria, yet lack MSI and have normal expression of mismatch repair proteins are common and clinically confusing. Little is known about the genetic alterations responsible for cancer in these families. Clinical heterogeneity in familial CRC and HNPCC was recognized early and a subgroup of patients were identified with an older age of cancer onset, MSS tumors located primarily in the left colon and lack of other cancer sites (21). Families such as these have been characterized more recently by Lindor et al who have defined these families that meet criteria for HNPCC via Amsterdam I with no mismatch repair deficiency as "familial colorectal cancer type X" (22). Families with colorectal cancer type X have been shown to have an increase in colorectal cancer risk only, and this risk is shown to be smaller than that in DMMR deficient families.

The family described in this paper is unlike those in the studies previously mentioned in that the incidence of endometrial cancer is high and other HNPCC malignancies such as ovarian cancer are represented. Llor et al prospectively studied families such as the one we describe that met Amsterdam I or II criteria and have none of the characteristic features of DMMR deficiency. They found that these families represent a high percentage (60%) of all HNPCC patients (20). Consistent with previous reports, average age of diagnosis of CRC was higher than in those with mutations in DMMR, and most cancers were located on the left side in contrast to the propensity of right-sided tumors in HNPCC. Families studied by Llor more often fulfilled Amsterdam II criteria, endometrial cancer prevalence was high, and there was a significant percentage of synchronous and metachronous adenomatous polyps and cancers.

Recommendations for those families with known or highly suspected Lynch syndrome are as follows (23). Colonoscopy is generally offered every 1-2 years starting at the age of 20 to 25 years or 10 years younger than the youngest age at diagnosis in the family. In women, annual endometrial biopsy and transvaginal ultrasound beginning at age 30 to 35 years are recommended. Additionally, urinalysis every 1-2 years beginning at age 25-35 as well as detailed physical exam with education and counseling regarding Lynch syndrome should be conducted every year past 21 years of age. Prophylactic surgery with a colorectal resection is acceptable for persons with a diagnosed cancer or polyp not resectable by colonoscopy. Hysterectomy or oophorectomy is an option after childbearing is completed as there is recent evidence for efficacy (24). Clear recommendations for genetic counseling, increased cancer surveillance and prevention in these families that fulfill Amsterdam criteria yet lack mutations in DMMR is unknown and demands further exploration. Professional organizations such as the American Cancer Society recommends colonoscopy every 5-10 years in these high risk families where colorectal cancer or polyps have been diagnosed prior to age 60 in any first-degree relative or in 2 or more first-degree relatives at any age (23). Although the molecular mechanism or specific gene contributing to the cancer phenotype is unknown, penetrance is high and these patients do warrant some form of increased surveillance; historically clinical judgement has been utilized to determine if Lynch syndrome screening guidelines are appropriate based on the cancer profile of the family.

The discovery of MLH1 methylation in the PBLs of one member of the family (III-4) with an early-onset colon cancer was unexpected. MLH1 methylation in normal tissues is infrequent. In our laboratory we have evaluated over 100 samples of DNA from PBLs of

women with endometrial cancer and found no other examples of methylation at the MLH1 locus. Sutter et al. screened 94 individuals with a high index of suspicion for genetic disease for MLH1 epimutation in PBLs. They found only two such examples which proved to be germline epimutations in both cases (23). Germline epimutation in individual III-4 is unlikely based on the lack of MLH1 methylation in other tissues (hair, bladder epithelium, buccal cells). The methylation in the PBLs of individual III-4 could be a sporadic event; however, it is appealing to suggest that it could mark an underlying predisposition to methylation contributing to his disease state or cancer susceptibility in the family.

In summary, this family represents an important group of patients with specific features, no evidence of DMMR deficiency, an autosomal dominant inheritance, and a high representation of endometrial cancer. The molecular basis of cancer susceptibility in this family is unknown. Future studies targeting families such as these as a distinct group are warranted and the need for genetic counseling and increased cancer surveillance should be addressed. Studies to evaluate a methylator phenotype in these families should also be considered as aberrant methylation may play a role in cancer susceptibility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Chen S, Wang W, Lee S, Nafa K, et al. Prediction of Germline Mutations and Cancer risk in the Lynch Syndrome. *JAMA* 2006;296(12):1479–87. [PubMed: 17003396]
2. Valle L, Perea J, Carbonell P, Fernandez V, Dotor A, et al. Clinicopathologic and Pedigree differences in Amsterdam-I positive Hereditary Nonpolyposis Colorectal Cancer Families according to tumor microsatellite instability status. *J Clin Oncol* 2007;25(7):1–6. [PubMed: 17146103]
3. Vasen HF, Mecklin JP, Khan PM, Lynch HT. The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer. *Dis Colon Rectum* 1991;34(5):424–5. [PubMed: 2022152]
4. Vasen HFA, Watson P, Mecklin JP, Lynch HP. New clinical criteria for hereditary non-polyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative Group on HNPCC. *Gastroenterology* 1999;116:1453–56. [PubMed: 10348829]
5. Rodriguez-Bigas MA, Boland CR, Hamilton SR, et al. A national cancer institute workshop on hereditary non-polyposis colorectal cancer syndrome: meeting highlights and Bethesda guidelines. *J Natl Ca Inst* 1997;89:1758–61.
6. Boland CR. Evolution for the nomenclature for the hereditary colorectal cancer syndromes. *Familial Cancer* 2005;4:211–218. [PubMed: 16136380]
7. Hampel H, Frankel W, Panescu J, Lockman J, et al. Screening for Lynch syndrome (Hereditary Nonpolyposis Colorectal Cancer) among endometrial cancer patients. *Cancer Res* 2006;66(15):7810–7817. [PubMed: 16885385]
8. Lindor NM, Peterson GM, Hadley DW, Kinney AY, Miesfelt S, et al. Recommendations for the care of individuals with an inherited predisposition to Lynch syndrome: A systematic review. *JAMA* 2006;296(12):1507–17. [PubMed: 17003399]
9. Dunlop MG, Farrington SM, Carothers AD, et al. Cancer risk associated with germline DNA mismatch repair gene mutations. *Hum Mol Genet* 1997;6(1):105–10. [PubMed: 9002677]
10. Peiffer SL, Herzog TJ, Tribune DJ, Mutch DG, Gersell DJ, Goodfellow PJ. Allelic loss of sequences from the long arm of chromosome 10 and replication errors in endometrial cancers. *Cancer Res* 1995;55:1922–1926. [PubMed: 7728760]

11. 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. [PubMed: 9071575]
12. Thibodeau SN, French AJ, Cunningham JM, Tester D, Burgart LJ, et al. Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. *Cancer Res* 1998;58(8):1713–18. [PubMed: 9563488]
13. Chen H, Taylor NP, Sotamaa KM, Mutch DG, Powell MA, et al. Evidence for heritable predisposition to epigenetic silencing of MLH1. *Int J Cancer* 2007;120(8):1684–88. [PubMed: 17230510]
14. Whelan A, Babb S, Mutch D, Rader J, Herzog T, Todd C, Ivanovich J, Goodfellow P. MSI in endometrial carcinoma: absence of MLH1 promoter methylation is associated with increased familial risk for cancers. *Int J Cancer* 2002;99:697–704. [PubMed: 12115503]
15. Simpkins SB, Bocker T, Swisher EM, Mutch DM, et al. MLH1 promoter methylation and gene silencing is the primary cause of microsatellite instability in sporadic endometrial cancer. *Human Mol Genet* 1999;8(4):661–66. [PubMed: 10072435]
16. Marsit CJ, Houseman EA, Christensen BC, Eddy K, Bueno R, et al. Examination of a CpG island methylator phenotype and implications of methylation profiles in solid tumors. *Cancer Res* 2006;66(21):10621–9. [PubMed: 17079487]
17. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci U S A* 1999;96:8681–6. [PubMed: 10411935]
18. Whitcomb BP, Mutch DG, Herzog TJ, Rader JS, et al. Frequent HOXA11 and THBS2 promoter methylation, and a methylator phenotype in endometrial adenocarcinoma. *Clin Cancer Res* 2003;9(6):2277–87. [PubMed: 12796396]
19. Lagerstedt Robinson K, Liu T, Vandrovcova J, Halvarsson B, et al. Lynch syndrome (hereditary nonpolyposis colorectal cancer) diagnostics. *J Natl Cancer Inst* 2007;99(4):291–9. [PubMed: 17312306]
20. Llor X, Pons E, Xicola R, Castells A, Alenda C, et al. Differential features of colorectal cancers fulfilling Amsterdam criteria without involvement of the mutator pathway. *Clin Cancer Res* 2005;11(20):7304–10. [PubMed: 16243801]
21. Jass JR, Cottier DS, Jeevarantam P, et al. Diagnostic use of microsatellite instability in hereditary non-polyposis colorectal cancer. *Lancet* 1995;346:1200–1. [PubMed: 7475662]
22. Lindor NM, Rabe K, Peterson GM, Haile R, Casey G, Baron J, et al. Lower cancer incidence in Amsterdam-I criteria families without mismatch repair deficiency: familial colorectal cancer type X. *JAMA* 2005;293(16):1979–85. [PubMed: 15855431]
23. Lindor NM, Petersen GM, Hadley DW, Kinney AY, et al. Recommendations for the care of individuals with an inherited predisposition to Lynch syndrome: A systematic review. *JAMA* 2007;296(12):1507–17. [PubMed: 17003399]
24. Schmeler KM, Lynch HT, Chen LM, et al. Prophylactic surgery to reduce the risk of gynecologic cancers in Lynch syndrome. *N Engl J Med* 2006;354:261–269. [PubMed: 16421367]
25. Suter CM, Martin DI, Ward RL. Germline epimutation of MLH1 in individuals with multiple cancers. *Nat Genet* 2004;36:497–501. [PubMed: 15064764]

Figure 1. Pedigree

Partial four-generation pedigree demonstrating clinical Lynch syndrome by fulfillment of Amsterdam II criteria. Lynch syndrome malignancies are represented across three generations. Asterisks represent the number of individuals for which DNA was available for genotyping.

Figure 2. Exclusion of MSH2 and MSH6 by linkage and normal expression in probands' tumors Haplotypes derived from PCR linkage studies of MSH2, D2S123, and MSH6 are demonstrated with divided bars. Haplotype analysis excludes linkage of MSH2 and MSH6 to cancer susceptibility. IHC of the two proband's tumors reveals normal expression of all DNA MMR proteins in family member II-3 and loss of MLH1 and PMS2 in family member II-5.

Figure 3. MLH1 methylation in normal PBL DNA

Panel A- Representation of MLH1 promoter and exon 1. Vertical black bars represent CpG pairs. The location of CpG sites evaluated in the three COBRA assays by restriction digest are shown. Assay 2 contains the previously documented critical CpG residues for gene expression.

Panel B-MLH1 COBRA for family members II-3, III-4, III-2 and a universally methylated control are presented demonstrating MLH1 methylation in individual III-4. PCR products were digested with BstUI and MboI.

Panel C-Bisulfite sequencing for individual III-4 demonstrating methylation of all 8 cytosine residues within the MLH1 promoter (shown by black arrows). Assay 2 from panel B was utilized to obtain bisulfite PCR products. Note that the reverse strand was sequenced.

Table 1

Tumor Characteristics

Patient	Sex	Diagnosis	Age at Diagnosis	IHC	MSI
I-2	F	Endometrial Ca	76	NA	NA
II-1	F	Colon Ca	72	normal	MSS
II-3	F	Endometrial Ca	82	normal	MSS
II-5	F	Endometrial Ca	67	MLH1 neg	MSI
		Colon Adenoma × 6	65(4), 67, 69	NA	NA
II-6	F	Colon Ca	48	NA	NA
II-7	F	Colon Adenoma × 3	72(2), 75	NA	NA
II-8	F	Ovary Ca	44	NA	NA
III-2	F	Colon Adenoma	57	Normal	NA
		Endo Hyperplasia	61	NA	NA
III-4	M	Colon Ca	54	NA	NA
		Colon Adenoma × 3	56(2), 57	normal	NA

MSS=Microsatellite Stable
MSI=Microsatellite Instability
NA=tissue not available