Original Article

Evidence for an hMSH3 Defect in Familial Hamartomatous Polyps

Sherry C. Huang, MD[1,2]; Jeffrey K. Lee, MD, MSc[3]; E. Julieta Smith, BS[3]; Ryan T. Doctolero, PharmD[3]; Akihiro Tajima, MD, PhD[3]; Stayce E. Beck, PhD[4]; Noel Weidner, MD[5]; and John M. Carethers, MD[3,4,6,7,8]

BACKGROUND: Patients with hamartomatous polyposis syndromes have increased risk for colorectal cancer (CRC). Although progression of polyps to carcinoma is observed, pathogenic mechanisms remain unknown. The authors examined whether familial hamartomatous polyps harbor defects in DNA mismatch repair (MMR), and assayed for somatic mutation of *PTEN*, a gene inactivated in the germline of some hamartomatous polyposis syndrome patients. **METHODS:** Ten hamartomatous polyposis syndrome patients were genotyped for germline mutations. Epithelial and nonepithelial polyp DNA were assayed for microsatellite instability (MSI) and *PTEN* frameshift mutation. DNA MMR and PTEN protein expression were assessed in all polyps by immunohistochemistry. In addition, 99 MSI-high sporadic CRCs and 50 each of $hMLH1^{-/-}$ and $hMSH3^{-/-}$ cell clones were examined for *PTEN* frameshifts. **RESULTS:** Twenty-five (58%) of 43 hamartomatous polyposis syndrome polyps demonstrated dinucleotide or greater MSI in polyp epithelium, consistent with *hMSH3* deficiency. MSI domains lost hMSH3 expression, and PTEN expression was lost in polyps from germline *PTEN* patients; sporadic hamartomatous polyps did not show any of these findings. *PTEN* analysis revealed wild type exon 7 and 8 sequences suggestive of nonexistent or rare events for *PTEN* frameshifts; however, MSI-high sporadic CRC showed 11 (11%) of 99 frameshifts within *PTEN*, with 4 tumors having complete loss of PTEN expression. Subcloning $hMLH1^{-/-}$ and $hMSH3^{-/-}$ cells revealed somatic *PTEN* frameshifts in 4% and 12% of clones, respectively. **CONCLUSIONS:** Nondysplastic epithelium from hamartomatous polyposis syndrome polyps harbors hMSH3 defects, which may prime neoplastic transformation. Polyps from $PTEN^{+/-}$ patients lose PTEN expression, but loss is not a universal early feature of all hamartomatous polyposis syndrome. However, *PTEN* frameshifts can occur in hMSH3-deficient cells, suggesting that hMSH3 deficiency could drive hamartomatous polyposis syndrome tumorigenesis. ***Cancer* 2011;117:492–500.** *© 2010 American Cancer Society.*

KEYWORDS: hamartomatous polyposis syndrome, microsatellite instability, colon cancer, PTEN hamartomatous tumor syndrome, DNA mismatch repair.

Hamartomatous polyposis syndromes are a group of clinically distinct disorders in which the predominant feature is multiple hamartomatous polyps in the gastrointestinal tract. These include juvenile polyposis syndrome (JPS), Peutz-Jeghers syndrome, and the PTEN hamartoma tumor syndrome, which encompasses Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome, Proteus syndrome, and Proteus-like syndrome. Polyps from these syndromes are composed of disorganized but normal cellular elements that demonstrate a distorted architecture. Despite the nondysplastic histological characteristics of these hamartomatous polyps, each of the hamartomatous polyposis syndromes carry specific cancer risks at different organ sites, including the colon.[1-4]

The cystic, inflammatory, and nondysplastic histological appearance of sporadic intestinal hamartomatous polyps suggests little or no risk for malignant transformation; however, the risk for malignant transformation of polyps in syndromic patients is high. For instance, patients with JPS have a 16% risk of developing colorectal cancer (CRC) when young, with a cumulative risk of 68% by age 60 years.[1,5] In Peutz-Jeghers syndrome, the cumulative risk of developing any type of cancer is 93% by the age of 65 years, inclusive of CRC.[6] Although the malignant potential of hamartomatous

Corresponding author: John M. Carethers, MD, Professor of Internal Medicine, University of Michigan, 3101 Taubman Center, 1500 E. Medical Center Drive, Ann Arbor, MI 48109; Fax: (734) 232-3838; jcarethe@umich.edu

[1]Department of Pediatrics, University of California at San Diego, San Diego, California; [2]Rady Children's Hospital, San Diego, California; [3]Department of Medicine, University of California at San Diego, San Diego, California; [4]Biomedical Sciences Program, University of California at San Diego, San Diego, California; [5]Department of Pathology, University of California at San Diego, San Diego, California; [6]Moores Comprehensive Cancer Center, University of California at San Diego, San Diego, California; [7]Veterans Administration Research Service, San Diego, California; [8]Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan

S.C.H. and J.M.C. designed research; S.C.H., J.K.L., E.J.S., R.T.D., A.T., S.E.B., and J.M.C. performed research; S.C.H., J.K.L., and J.M.C. analyzed data; N.W. provided some pathology specimens; S.C.H., J.K.L., and J.M.C. wrote the paper. All authors approved the final version of the article.

DOI: 10.1002/cncr.25445, **Received:** December 23, 2009; **Revised:** March 25, 2010; **Accepted:** April 19, 2010, **Published online** September 15, 2010 in Wiley Online Library (wileyonlinelibrary.com)

polyps in Peutz-Jeghers syndrome has been debated, a recent case report demonstrated a sequence of hamartoma-dysplasia-carcinoma in a patient with Peutz-Jeghers syndrome.[7] In addition, among the different PTEN hamartoma tumor syndrome disorders, cancer risk has been clearly documented in patients with Cowden syndrome,[8] particularly with breast, thyroid, and endometrial cancer.[9,10] The risk of CRC associated with Cowden syndrome is not well characterized; however, 1 case report described a patient with Cowden syndrome who had a *PTEN* mutation and metachronous colon carcinoma, both of which appeared to arise from within the hamartomatous polyp.[11]

In several examples of patients with JPS, malignancy arises directly from the intestinal juvenile polyps.[1,4,12] Because some polyps have adenomatous epithelium, even in patients as young as 3 years,[12] it has been suggested that the development of malignancy may be based on the characteristic adenoma-carcinoma sequence.[13,14] In the model proposed by Goodman, progression from juvenile hamartomatous polyps takes place through an intermediate "juvenile polyp with adenomatous features" before the appearance of carcinoma, although the genetic mechanisms that drive this process are not known.[2] However, it does suggest that the final pathway to CRC is at the epithelial level, and not the stromal level.[2,4]

There are at least 2 well-described pathways for CRC pathogenesis, highlighted in their extreme sense by familial adenomatous polyposis (FAP) and Lynch syndrome. Chromosomal instability is observed in tumors from FAP patients, in which cytogenetic alterations such as chromosome breaks, duplication, rearrangements, and deletions form an aneuploid tumor, and occurs in 80% to 85% of sporadic CRC cases.[13] Microsatellite instability (MSI), caused by defective DNA mismatch repair (MMR), is observed in patients with Lynch syndrome and is caused by a germline mutation in DNA MMR genes. In addition, MSI is observed in 15% to 20% of sporadic CRC because of hypermethylation of the *hMLH1* gene promoter.[13,15-17] In particular, the repair spectrum of specific DNA MMR proteins predicts the phenotype of Lynch syndrome. For example, patients with *hMSH2* and *hMLH1* mutations have early onset presentation, whereas patients with *hMSH6* mutations have a later onset of presentation.[13,18] The redundancy of hMSH6 and hMSH3 functions, with hMSH6-hMSH2 heterodimers repairing single nucleotide mispairings and 1 to 2 nucleotide insertion-deletion loops, and hMSH3-hMSH2 heterodimer repairing $\geq$2 nucleotides insertion-deletion loops, helps moderate the clinical phenotype and age of presentation for CRC compared with *hMSH2* or *hMLH1* mutations in Lynch syndrome. Misrepair when hMSH6 or hMSH3 functions are defective in any human tissue will leave the specific genetic signature of single insertion-deletion loop mistakes or larger insertion-deletion loop mistakes, respectively.[13,19]

In this study, we explored if 1 of the 2 main pathways of genomic instability operative in CRC could be operative in familial hamartomatous polyps as a mechanism for transformation. We discovered that the normal, nondysplastic epithelium of familial hamartomatous polyps harbors defects in DNA MMR consistent with an hMSH3 deficit. We also demonstrate that this defect could inactivate *PTEN* through mutation of its coding hexadenine tract, demonstrating a potential mechanism for somatic inactivation of *PTEN* that could contribute to neoplastic formation.

MATERIALS AND METHODS

Patient Materials

Ten patients were previously evaluated and identified by pediatric and adult gastroenterologists to have a hamartomatous polyposis syndrome. Three patients demonstrated clinical findings consistent with Bannayan-Riley-Ruvalcaba syndrome. One patient was diagnosed with Cowden syndrome with the development of intestinal hamartomas, cutaneous lipomas, and a history of thyroid adenoma. The remaining 6 (1 set of identical twins) patients presented with only intestinal hamartomatous polyposis and were given the initial diagnosis of JPS (Table 1). Sporadic, nonsyndromic hamartomatous polyps (n = 12) were used for comparison. Sporadic patients had only 1 polyp at presentation that was removed by polypectomy on colonoscopic evaluation.

Ninety-nine colon cancer tumors previously found to have MSI-high from 3 sporadic cohorts available to the authors were used for somatic frameshift *PTEN* mutational analysis.[20-22]

This study was approved by the human subjects protection programs at University of California at San Diego and the San Diego Veterans Administration Healthcare system.

Genotyping Hamartomatous Syndrome Patients

Informed consent was obtained for genetic testing, and institutional review board approval was obtained for tissue evaluation. Genomic DNA was extracted from whole

Table 1. Patient Characteristics and Germline Mutations, and Results of Their Polyps Examined for Microsatellite Instability

Patient	Age, y	Diagnosis	Clinical Findings	Germline Mutation (Site)	Polyps With MSI /Total Polyps Examined	Cystic or Surface Epithelial Domains With MSI/Total Epithelial Domains	Lamina Propria Domains With MSI/Total Lamina Propria Domains
1	10	BRRS	Macrocephaly, intestinal polyps, pigmented spotting of pubis, DD	*PTEN* (del 10q23.2-10q24.1)	5/14	5/29	0/12
2	12	BRRS	Macrocephaly, intestinal polyps, DD	*PTEN* (del 10q23.1-10q24.2)	4/6	4/13	0/6
3	14	JPS	Intestinal polyps	*PTEN* (exon 4 splice site mutation)	2/2	2/4	0/2
4	14	JPS	Intestinal polyps	*PTEN* (exon 4 splice site mutation)	3/3	3/6	0/3
5	26	CS	Intestinal polyps, thyroid adenoma, cutaneous lipomas	*PTEN* (R129X)	3/4	4/9	0/4
6	12	JPS	Intestinal polyps	WT	1/2	1/3	0/1
7	24	JPS	Intestinal polyps	WT	1/2	1/4	0/2
8	2	BRRS	Intestinal polyps, AV canal, macrocephaly, DD	WT	3/6	3/6	0/3
9	28	JPS	Intestinal polyps	Not performed	1/2	1/3	0/1
10	24	JPS	Intestinal polyps	*BMPR1A* (T78I)	2/2	4/4	0/2

MSI indicates microsatellite instability; BRRS, Bannayan-Riley-Ruvalcaba Syndrome; JPS, juvenile polyposis syndrome; CS, Cowden syndrome; DD, developmental delay; WT, wild type; AV, atrioventricular.

blood lymphocytes from the hamartomatous patients using phenol-chloroform for phase extraction and subsequent ethanol precipitation. All exons for *PTEN* were analyzed by polymerase chain reaction (PCR) as described previously.[23] The amplicons were gel purified and then cycle-sequenced using the Big Dye Terminator chemistry (PE Applied Biosystems, Foster City, Calif). Consequently, the product was filtered through a Sepharose column (Princeton Separations, Adelphia, NJ), and subjected to analysis in an Applied Biosystems 310 Genetic Analyzer. Mutational analysis was performed by direct comparison with the wild type genetic sequences for each exon (Whitehead Institute Gene Bank). In some cases, patient blood was sent for commercial genotyping. In addition to *PTEN*, germline mutations in *SMAD4* and *BMPR1A* were assessed in the cohort.

Microsatellite Instability Analysis on Hamartomatous Polyps

Regions of tissue were microdissected from paraffin-embedded, formalin-fixed tissue of the polyps of all hamartomatous patients. We microdissected multiple cystic epithelial, surface epithelial, and lamina propria domains from polyps (Table 1). Extracted DNA was purified by proteinase K digestion and phenol-isoamyl-alcohol chloroform phase extraction. The DNA was then amplified by PCR at microsatellite loci that have previously been recommended by a National Cancer Institute panel, and included BAT25, BAT26, D2S123, D5S345, and D17S250.[24] We also used other dinucleotide markers, including 1 intronic at *hMSH2*, D5S107, D10S1765, D10S1687, and D3S1611, and tetranucleotide markers including *MYCL1* and UT764. Primer sequences and conditions of the PCR assay and gel electrophoresis have been described previously.[19,21,22,25]

PTEN Exon 7 and 8 Mutational Analysis

DNA from hamartomatous polyp sections (stromal and glandular epithelium) used in the MSI analysis, or DNA from MSI-high colon cancers and colon cancer cell clones, were amplified by PCR with flanking intronic regions for *PTEN* exon 7 and exon 8 to investigate mutations in the microsatellite region of both exons by MSI analysis as described above. DNA was also subjected to sequencing analysis using the Big Dye Terminator chemistry (PE Applied Biosystems) to verify frameshift mutation.

Immunohistochemical Analysis

Patient polyps were analyzed for PTEN, hMSH2, hMLH1, hMSH6, and hMSH3 expression using immunohistochemical staining. Solitary nonsyndromic juvenile polyps were used as controls. Paraffin sections of the polyp material were deparaffinized in xylene and rehydrated in graded alcohols to water. Slides were immersed in sodium citrate buffer (ph 6.0) and heat-treated for antigen retrieval. Slides were processed using a DAKO Signal Catalyzed Amplification System (DAKO, Carpinteria, Calif). Endogenous peroxidase activity was blocked by incubating with 3% H_2O_2. Goat serum (5%) was added for 1 hour to block nonspecific protein binding. Slides were incubated overnight with primary antibody to PTEN at 1:125 dilution (Mouse Monoclonal IgG, Santa Cruz Biotechnology, Santa Cruz, Calif), hMSH2 at 1:200 dilution (Mouse Monoclonal IgG, Santa Cruz Biotechnology), hMLH1 at 1:150 dilution (Mouse Monoclonal IgG, Santa Cruz Biotechnology), hMSH6 at 1:250 dilution (Mouse Monoclonal IgG, Serotec, Raleigh, NC), and hMSH3 at 1:50 dilution (polyclonal antibody, gift from Drs. Josef Jiricny and Giancarlo Marra, Geneva, Switzerland), then rinsed with 0.1% tris-buffered saline (TBS)-Tween 20. Biotinylated secondary antibody was added for 15 minutes, followed by incubation with peroxidase-labeled streptavidin for 15 minutes at room temperature. Sections were washed with TBS-Tween 20, incubated with diaminobenzidine and H_2O_2 for 1 minute, lightly counterstained with hematoxylin, dehydrated in graded alcohols, cleared with xylene, and coverslipped. Polyp material from the MSI-high cancers with evidence for *PTEN* gene frameshift mutation was also subjected to immunohistochemical analysis for somatic PTEN expression.

Cell Lines

Three colon cancer cell lines were used to characterize *PTEN* mutations. These colon cancer cells lines were used because no suitable hamartomatous polyposis cell line exists for evaluation. Cell lines used include HCT116 cells, which are an MSI-high cell line because of biallelic mutations in the *hMLH1* gene and secondary somatic biallelic mutations in *hMSH3*. We also used HCT116 cells complemented with chromosome 3, which are microsatellite stable at single base pair mismatches because of *hMLH1* complementation, but retain biallelic *hMSH3* mutations.[26] The third cell line used was SW480, which is completely mismatch repair-proficient. Cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Carlsbad, Calif) with 10% fetal bovine serum. For HCT116 + Ch3 cells, 400 μg/mL of G418 (GIBCO BRL, Gaithersburg, Md) was added. All cell lines were cultured at 37°C in 5% CO_2.

Cell DNA TA Subcloning and Sequencing

Cells were trypsinized, cell pellets were lysed, and DNA was purified as above. The oligomers used for amplification of exon 7 of *PTEN* were 5′-CGACGGGAAGA CAAGTTCAT-3′ and 5′-AGGTTTCCTCTGGTCC TGGT-3′ and for exon 8 of *PTEN* were 5′GCGTGC AGATAATGACAAGG-3′ and 5′-TGGATTTGACGG CTCCTCTA-3′. PCR products were cloned to separate individual allelic sequences by using the TA cloning kit (Invitrogen) according to the manufacture's instructions. DNA from 50 clones per cell line was extracted using a miniprep kit (Life Technologies, Rockville, Md). The DNA was then subjected to sequencing analysis.

RESULTS

Germline Mutations of Patients With Hamartomatous Polyposis Syndromes

We evaluated 10 patients who were clinically found to have a hamartomatous polyposis syndrome (Table 1). Three patients were diagnosed with Bannayan-Riley-Ruvalcaba syndrome, 1 were diagnosed with Cowden syndrome, and 6 were found to have only intestinal hamartomas, fitting the clinical diagnosis of JPS. Of the 6 JPS patients, 3 were adult age at diagnosis, whereas the remaining 3 were teenagers. Although the penetrance of Cowden syndrome approaches 90% by age 20 years, the 3 teenagers with only intestinal hamartomatous polyposis could eventually develop symptoms consistent with Cowden syndrome; however, they were classified as having JPS for the purposes of this study. Two Bannayan-Riley-Ruvalcaba syndrome patients, twin JPS patients (Patients 3 and 4, Table 1), and the Cowden syndrome patient were all found to have mutations in the *PTEN* gene.[23,27] The Cowden syndrome patient was found to have a C to T transition at nucleotide 388 of *PTEN*, effectively altering an arginine to a stop codon (R129X). This is a well-described nonsense mutation in Cowden syndrome and is thought to abrogate PTEN phosphatase function. The JPS twins were found to have an intronic deletion at a critical pyrimidine tract at the transition between intron 3 and exon 4.[23] This mutation causes abnormal splicing between exons 3 and 4 and potentially alters PTEN phosphatase function.[23] One patient diagnosed with JPS was found to carry a germline mutation in *BMPR1A*.[28] DNA

from 3 of the 4 remaining patients was sequenced for mutations in *PTEN* and *SMAD4* and the dominant mutations in *BMRP1A* (1 patient was not available for DNA analysis). No germline mutations were found in the remaining 3 patients (all were JPS patients).

Epithelial Components From Familial Hamartomatous Polyps Demonstrate a DNA Mismatch Repair Defect Consistent With hMSH3 Deficiency

We performed microdissection followed by microsatellite analysis on domains from familial hamartomatous polyps in an attempt to characterize the genetic pattern that could drive neoplastic pathogenesis or transformation. The numbers of domains (consisting of surface epithelium, cystic epithelium, and lamina propria) as well as the number of polyps examined are listed in Table 1. Surprisingly, some of the epithelial domains (exclusively cystic or surface epithelium) from polyps of all 10 patients demonstrated MSI-high, defined as $\geq$40% of the markers showing a novel allele. This finding suggests that inactivation of DNA MMR within the normal-appearing epithelium is a potential mechanism for neoplastic progression. Of 43 polyps examined from the 10 patients, 25 (58%) polyps had evidence for MSI in at least 1 microdissected domain (Table 1), which was uniformly an epithelial domain and not a lamina propria domain. In analyzing the microsatellite mutation pattern, we noticed that in all cases, only dinucleotide repeats showed novel alleles, with no new allele at mononucleotide markers (Fig. 1A, B). This pattern suggested an hMSH3 defect, because hMSH6 defects are consistent with mononucleotide (single insertion-deletion loops) defects, and hMLH1 and hMSH2 defects would cause mutation in both mononucleotide and dinucleotide markers.[13,19] To verify an hMSH3 defect, we performed microsatellite analysis at tetranucleotide microsatellite repeats, which confirmed the dinucleotide findings in the same domains (Fig. 1C). To assess this finding further, we performed immunohistochemistry using hMSH2, hMLH1, hMSH6, and hMSH3 antibodies on the familial hamartomatous polyps. Only hMSH3, but not hMLH1, hMSH6, or hMSH2, was lost from the affected epithelium of these polyps (Fig. 2). These findings indicate that an hMSH3 deficit is present in the nondysplastic and normal-appearing epithelial components of familial hamartomatous polyps, and could be a potential mechanism that drives neoplastic transformation. This hMSH3 defect was not observed in sporadic juvenile polyps tested for MSI and hMSH3 immunohistochemistry.

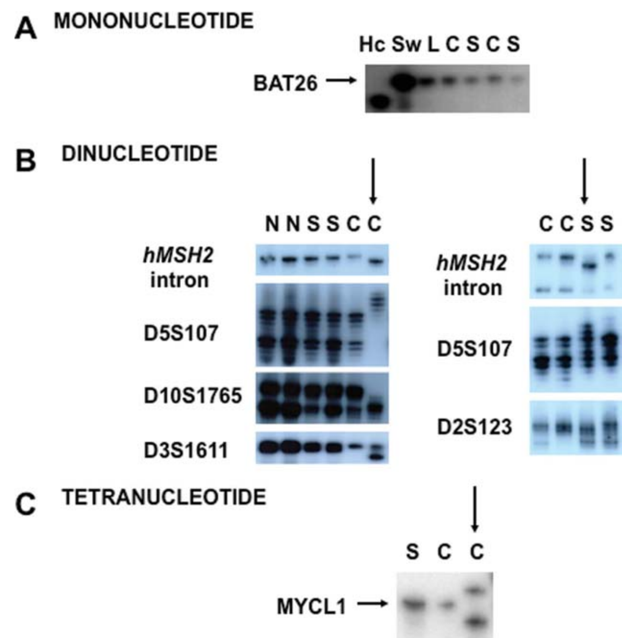

Figure 1. Microsatellite alterations in familial hamartomatous polyps are shown. (A) An example is shown of 1 familial hamartomatous polyp assessed at 5 microdissected domains (L = lamina propria, C = cystic epithelium, S = surface epithelium) with 2 cell line controls (Hc = HCT116, a mismatch repair [MMR]-deficient cell line and Sw = SW480, a MMR-proficient cell line) for mononucleotide microsatellite instability (MSI). Note in the HCT116 lane that the *BAT26* mononucleotide is shortened, but SW480 and all other familial hamartomatous polyp domains, as well as domains from other polyps, showed wild type length for *BAT26*. (B) Two examples are shown of familial hamartomatous polyps from 2 separate patients with MSI-high at several dinucleotide microsatellites. Six microdissected domains from 1 polyp are shown in the left panel (N = normal colon, S = surface epithelium, C = cystic epithelium), and 4 microdissected domains from 1 polyp are shown in the right panel. Vertical arrows denote domains with MSI. (C) Example of 1 familial hamartomatous polyp assessed at 3 microdissected domains (S = surface epithelium, C = cystic epithelium) at *MYCL1*, a tetranucleotide microsatellite. Vertical arrow denotes domain with MSI.

PTEN Expression Is Lost From the Epithelium of Familial Hamartomatous Polyps in Patients With a PTEN Germline Mutation, and Is Mutated in MSI-High Colorectal Cancers

Patients with PTEN hamartoma tumor syndrome have a germline mutation within the *PTEN* gene, and presumably the nonmutant allele becomes inactivated within their polyps as a way to completely abrogate PTEN's tumor suppression function. We performed immunohistochemistry for PTEN on polyps from all 10 hamartomatous

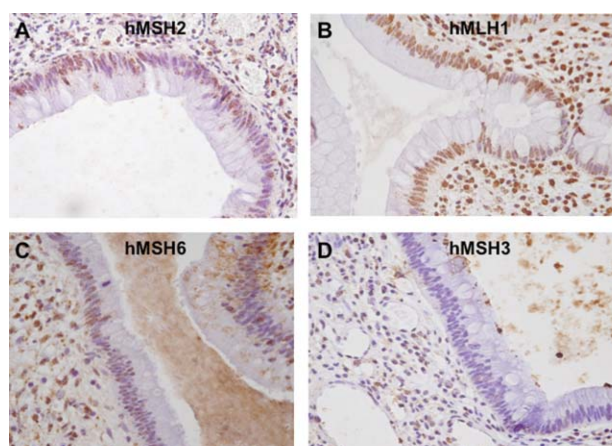

Figure 2. Examples are shown of DNA mismatch repair protein expression by immunohistochemistry in familial hamartomatous polyps. (A) hMSH2 expression is shown. Note the presence of hMSH2 in the nucleus. This protein was present in all microdissected domains. (B) hMLH1 expression is shown. hMLH1 is abundantly present in the nucleus. This protein was present in all microdissected domains. (C) hMSH6 expression is shown. Like hMSH2, hMSH6 is faintly positive in the nucleus. This protein was present in all microdissected domains. (D) hMSH3 expression is shown. In microdissected domains that demonstrated dinucleotide or greater microsatellite instability, hMSH3 was absent.

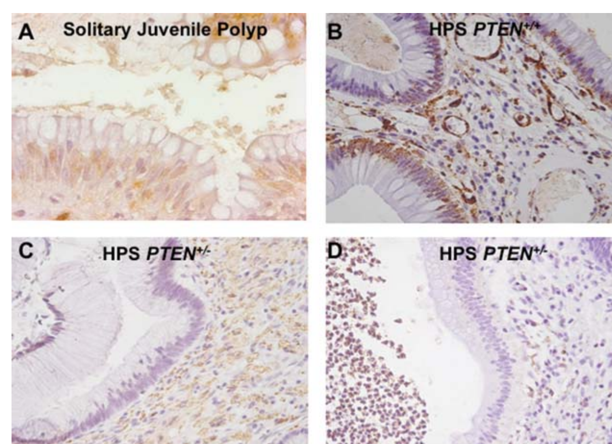

Figure 3. Examples are shown of PTEN protein expression by immunohistochemistry in familial hamartomatous polyps. (A) PTEN expression is shown in a solitary, nonfamilial juvenile polyp showing some expression in the epithelium. (B) PTEN expression is shown in a familial hamartomatous polyp from a patient genotyped as *PTEN* wild type. Note the expression of PTEN in the epithelium. (C, D) Two different familial hamartomatous polyps stained for PTEN are shown from 2 individuals who are genotyped heterozygous mutant for *PTEN*. Note the loss of PTEN expression from the epithelium in these 2 polyps. HPS indicates hamartomatous polyposis syndrome.

syndrome patients as well as 12 control solitary, nonfamilial juvenile polyps. Stromal cells exhibited a brown staining in the cytoplasm, indicating presence of the PTEN protein (Fig. 3). Complete absence of cytoplasmic protein was noted in the cystic epithelium in polyps from all 5 patients who had demonstrated a germline *PTEN* mutation (Fig. 3C, D). PTEN expression was seen in the cystic epithelium of hamartomatous polyposis patients genotyped as wild type *PTEN*, as well as in all control sporadic juvenile polyps (Fig. 3A, B). This finding suggests that loss of PTEN is not universal in all hamartomatous polyps, and although *PTEN* is a germline cause for hamartomatous polyp formation (as is *BMPR1A, SMAD4, STK11*, and *ENG* mutations),[4] *PTEN* may not be an early key factor for neoplastic transformation. To assess this further, we examined the epithelial domains taken from the 5 patients with germline *PTEN* mutation for frameshift mutation of *PTEN* at 1 or both of its coding hexadenine (A_6) tracts in exon 7 and 8 of *PTEN*. From these domains, we failed to find any *PTEN* frameshift mutation, suggesting that although hMSH3 may be lost from epithelial components, mutational inactivation of *PTEN* by hMSH3 may not be common. This absence of PTEN frameshift mutation in hMSH3 deficiency would not be totally unexpected, given the repair profile of hMSH3-hMSH2 heterodimers on noncoding microsatellites.[13,19] Alternatively, the occurrence of frameshift mutation in *PTEN*, particularly in the setting of hMSH3 deficiency, may be an extremely rare event.

To test this last hypothesis, we examined a collection of 99 sporadic MSI-high CRCs, most of which would be expected to have hypermethylation of *hMLH1* causing a complete deficit of DNA MMR, and this would exhibit stronger mutational pressure than hMSH3-deficient hamartomatous polyp epithelium. *PTEN* microsatellite analysis of exons 7 and 8, followed by DNA sequencing, revealed 2 (2%) of 99 samples with a mutation in exon 7 and 9 (9%) of 99 samples with a mutation in exon 8 (Fig. 4A) in these MSI-high tumors (Table 2). Immunohistochemistry for PTEN on the *PTEN*-mutant, MSI-high tumors showed 4 (44%) of 9 of exon 8 mutants lost expression of PTEN (Fig. 4B), whereas the 2 exon 7 mutants had only diminished PTEN expression. Indeed, PTEN expression was lost during the adenoma-to-carcinoma progression in 1 MSI cancer that had the various stages present on 1 pathological slide (Fig. 4B). None of the wild type *PTEN* samples in this MSI-high CRC group showed loss of PTEN expression. Taken together, these data suggest that PTEN expression is lost in the epithelium of polyps from PTEN hamartoma tumor syndrome patients by an unclear mechanism that may or may not be driven by an hMSH3 defect and may not be

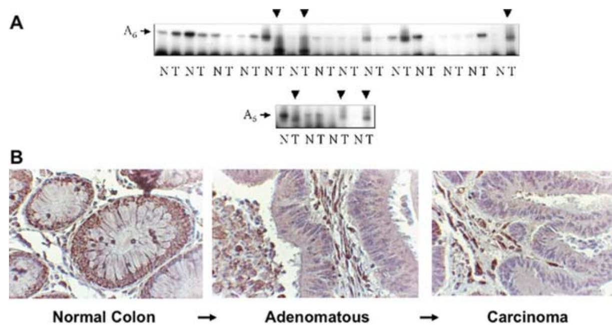

Figure 4. *PTEN* frameshift mutations and PTEN expression are shown in microsatellite instability (MSI)-high sporadic colorectal cancers. (A) Microsatellite analysis of 18 normal (N) and tumor (T) pairs for *PTEN* exon 8 frameshift mutation is shown. Arrowheads indicate frameshifts within tumors from the wild type A_6 sequence within exon 8 of *PTEN*. (B) Immunohistochemistry for PTEN expression in a single case of MSI-high colon cancer shows reduction of PTEN expression from normal glands (left panel) to adenomatous glands (middle panel) to near complete loss of PTEN expression in the adenocarcinoma (right panel).

Table 2. Prevalence of *PTEN* Frameshift Mutations in 99 Microsatellite Instability-High Sporadic Colorectal Cancers

PTEN Exon 7 Mutations	*PTEN* Exon 8 Mutations
2/99 (2%)	9/99 (9.1%)

important in the early pathogenesis of non-PTEN hamartoma tumor syndrome patients. As demonstrated in MSI-high CRC, complete inactivation of PTEN in the setting of a complete DNA MMR defect (*hMLH1*$^{-/-}$) is seen in only 4 (4%) of 99 cancers, which suggests that an hMSH3 defect would be more subtle in driving frameshift mutation.

PTEN Coding Frameshift Mutations Occur in hMSH3-Deficient Cells

To further examine the possibility that *hMSH3* deficiency could drive *PTEN* frameshift mutation as a way to somatically inactivate PTEN, we used human colon cancer cell lines that contained defined MMR defects, as there are no known hamartomatous cell lines. HCT116 colon cancer cells harbor *hMLH1* and *hMSH3* defects, whereas HCT116 + Ch3 cells have been complemented with 1 copy of human chromosome 3, the location of *hMLH1*, and harbor only *hMSH3* defects.[26] SW480 cells contain fully functional DNA mismatch repair. To assess for *PTEN* frameshift mutations, we subcloned each cell line by limiting dilution, and expanded 50 clones from each cell line for *PTEN* mutational analysis by sequencing. As expected, all MMR-proficient SW480 clones demonstrated wild type *PTEN* hexadenine tracts at both exons 7 and 8. In *hMLH1*-defective HCT116 cells, no mutations were observed for exon 7, but 2 (4%) of 50 clones had an insertion mutation ($A_6 \rightarrow A_7$) in exon 8 of *PTEN* (Table 3). This frequency of mutation is slightly lower than that observed in our MSI-high CRC cohort. Surprisingly, *hMSH3*-defective HCT116 + Ch3 cells also demonstrated exon 8 mutations (5 $A_6 \rightarrow A_7$ and 1 $A_6 \rightarrow A_5$) in 6 (12%) of 50 clones, with no mutation found in exon 7 of *PTEN* (Table 3). These data indicate that frameshift mutation of *PTEN*, particularly the exon 8 hexadenine coding tract, could be susceptible to mutation in the setting of hMSH3 deficiency.

Table 3. Prevalence of *PTEN* Frameshift Mutations in Colon Cancer Cell Lines With Known DNA MMR Defects

Cells	*PTEN* Exon 7 Mutations	*PTEN* Exon 8 Mutations
HCT 116 cells (*hMLH1*$^{-/-}$, *hMSH3*$^{-/-}$)	0/50 (0%)	2/50 (4%)[a]
HCT 116 + Ch3 cells (*hMLH1* restored, *hMSH3*$^{-/-}$)	0/50 (0%)	6/50 (12%)[b]
SW480 cells (MMR proficient)	0/50 (0%)	0/50 (0%)

MMR indicates mismatch repair.
[a] 2/50 showed insertions.
[b] 5/50 showed insertions and 1/50 showed deletion.

DISCUSSION

Hamartomatous polyposis syndromes share a common feature of intestinal hamartomatous polyps. Each syndrome has cancer-specific risks associated with its phenotype.[4] Colorectal cancer in particular is associated with many of these syndromes, and although a clear progression from hamartomatous polyp to cancer has been hypothesized, it has not been mechanistically demonstrated. In this study, we demonstrate the following findings: 1) nondysplastic epithelial components of most familial hamartomatous polyps demonstrate MSI-high in a pattern consistent with loss of function of hMSH3; 2) hMSH3 is absent from these same epithelial domains; 3) a potential target of loss of DNA MMR, *PTEN*, is absent from the epithelial components of polyps from patients with a *PTEN* germline mutation; 4) in the epithelial domains tested from PTEN hamartoma tumor syndrome polyps, we could not identify frameshift mutation of *PTEN*, but could demonstrate this in a panel of MSI-high sporadic CRC with observed progressive PTEN loss in the adenoma-to-carcinoma sequence; and 5) cells with an *hMSH3* defect can accumulate mutations in *PTEN*, particularly at *PTEN*'s exon 8 microsatellite. We suggest that the observed hMSH3 defect is a potential mechanism for

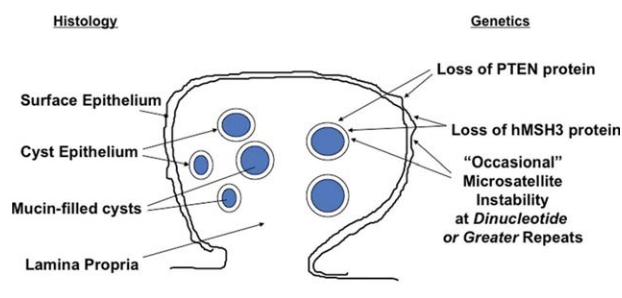

Figure 5. A schematic summarizes genetic observations in familial hamartomatous polyps. The pattern of occasional microsatellite instability (MSI)-high at dinucleotide or greater microsatellite sequences suggested a genetic signature consistent with loss of hMSH3 function; indeed, hMSH3 expression was lost in microdissected domains that showed the MSI. The loss of PTEN expression is mainly observed in those with germline *PTEN* mutations; however, the rare incidence of *PTEN* frameshifts (or other gene frameshifts) in the setting of loss of hMSH3 function cannot be completely ruled out.

neoplastic transformation in familial hamartomatous polyps, but likely occurs subtly and rarely when compared with an hMSH2 or hMLH1 defect. A schematic of a familial hamartomatous polyp is presented in Figure 5.

Our data indicate the novel finding of a DNA MMR defect within normal-appearing cells from familial hamartomatous polyps. In this regard, histological analysis does not match the genetic or biochemical (ie, microsatellite mutation pattern) findings. In a previous example, limiting dilution of lymphocytes from Lynch syndrome patients who are histologically normal can demonstrate DNA MMR defects.[29] This finding also suggests that the DNA MMR defects precede any dysplastic changes, an important observation that might describe the earliest neoplastic trigger for adenomatous transformation. In Lynch syndrome, where about 66% of polyps show MSI-high,[13,30] it is believed that neoplastic transformation came after the initial second hit to the remaining wild type MMR gene allele to fully inactivate DNA MMR.

The MMR protein hMSH3 is thought to repair insertion-deletion loops of $\geq$2 nucleotides,[13,19] although this observation has been challenged recently.[31] The spectrum of microsatellite mutations would be expected to be dinucleotide (insertion-deletion loops of 2) over mononucleotide microsatellites, as we observed, and in "elevated microsatellite alteration at selective tetranucleotide repeats,"[19] which we also observed in our data. Although *PTEN* contains only mononucleotide microsatellites, it is entirely possible that DNA polymerase slippage can create insertion-deletion loops of 2 adenines (instead of 1 adenine), which could involve hMSH3 for repair. This could be a very rare event (as compared with an insertion-deletion loop of 1 adenine, which involves hMSH6 over hMSH3 function) and hard to detect without large-scale analyses. Our lack of observation in the relatively few domains from the familial hamartomatous polyposis could be explained by this, with a slight increase in detection by our subcloning approach of MMR-defective cancer cells. In addition, yeast studies recently revealed that the MSH3-MSH2 heterodimer could repair specific base-base mispairs under certain conditions, a spectrum that had only been attributed to hMSH6-hMSH2 heterodimers.[31]

PTEN is a tumor suppressor protein with lipid and protein phosphatase activity. Germline mutations in *PTEN* cause PTEN hamartoma tumor syndrome, and we and others demonstrate complete inactivation of PTEN expression in the epithelium of their hamartomatous polyps.[32] Hamartomatous polyposis syndrome patients without *PTEN* germline mutations, however, do not show complete loss of PTEN, suggesting that PTEN may not be a common driving force for early neoplastic transformation in these polyps. PTEN inactivation by DNA MMR is common in endometrial cancers and prostate cancers that show MSI-high, and has been previously reported to be mutated in up to 19% of MSI-high CRC.[33] Thus, although *PTEN* is a clear target for frameshift mutations in certain tumors and drives their pathogenesis, it potentially can be mutated in hamartomatous polyps but may not drive their early transformation. We acknowledge that the correlation of *PTEN* inactivation with hMSH3 deficiency is not definitive with this study.

In summary, histological normal-appearing epithelial components of familial hamartomatous polyposis demonstrate a defect in DNA MMR, namely hMSH3 function. We demonstrate that an hMSH3 defect can be associated with frameshift mutation of *PTEN*, but other genes could be targets for mutation as well. An hMSH3 defect is predicted to be mild compared with other DNA MMR defects because of redundancy with hMSH6 function, and no germline *hMSH3* mutation has ever been found as a cause of Lynch syndrome.[13,34] However, our findings suggest a potential mechanism for neoplastic transformation for hamartomatous polyps, which have been suggested to progress through a hamartoma → adenoma → carcinoma sequence.[2] Loss of hMSH3 could occur in the setting of inflammation, a common finding within hamartomatous polyps, and inflammation has been associated with acquired DNA MMR defects.[35–37] These findings may explain, in part, an increased neoplastic risk within the colon in these syndromes.

CONFLICT OF INTEREST DISCLOSURES

Supported by the US Public Health Service (DK064560 to S.C.H. and DK067287 and CA90231 to J.M.C.), the University of California at San Diego Digestive Diseases Research Development Center (DK080506), the Foundation for Digestive Health and Nutrition (Research Scholar Award to S.C.H.), and the Veterans Administration Research Service (Merit Review Award to J.M.C.).

REFERENCES

1. Jass JR, Williams CB, Bussey HJ, et al. Juvenile polyposis—a precancerous condition. *Histopathology.* 1988;13:619-630.
2. Goodman ZD, Yardley JH, Milligan FD. Pathogenesis of colonic polyps in multiple juvenile polyposis. Report of a case associated with gastric polyps and carcinoma of the rectum. *Cancer.* 1979;43:1906-1913.
3. Sassatelli R, Bertoni G, Serra L, et al. Generalized juvenile polyposis with mixed pattern and gastric cancer. *Gastroenterology.* 1993;104:910-915.
4. Carethers JM. Unwinding the heterogeneous nature of hamartomatous polyposis syndromes. *JAMA.* 2005;294:2498-2500.
5. Heiss KF, Schaffner D, Ricketts RR, Winn K. Malignant risk in juvenile polyposis coli: increasing documentation in the pediatric age group. *J Pediatr Surg.* 1993;28:1188-1193.
6. Giardiello FM, Brensinger JD, Tersmette AC. Very high risk of cancer in familial Peutz-Jeghers syndrome. *Gastroenterology.* 2000;119:1447-1453.
7. Bouraoui S, Azouz H, Kechrid H. Peutz-Jeghers' syndrome with malignant development in a hamartomatous polyp: report of 1 case and review of the literature. *Gastroenterol Clin Biol.* 2008;e32:250-254.
8. Zbuk KM, Eng C. Cancer phenomics: RET and PTEN as illustrative models. *Nat Rev Cancer.* 2007;7:35-45.
9. Starink TM, van der Veen JP, Arwert F. The Cowden syndrome: a clinical and genetic study in 21 patients. *Clin Genet.* 1986;29:222-233.
10. Eng C. Will the real Cowden syndrome please stand up: revised diagnostic criteria. *J Med Genet.* 2000;37:828-830.
11. Bosserhoff AK, Grussendorf-Conen EI, Rubben A. Multiple colon carcinomas in a patient with Cowden syndrome. *Int J Mol Med.* 2004;18:643-647.
12. Giardiello FM, Hamilton SR, Kern SE, et al. Colorectal neoplasia in juvenile polyposis or juvenile polyps. *Arch Dis Child.* 1991;66:971-975.
13. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. *Gastroenterology.* 2008;135:1079-1099.
14. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell.* 1990;61:759-767.
15. Herman JG, Umar A, Polyak K, et al. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc Natl Acad Sci USA.* 1998;95:6870-6875.
16. Veigl ML, Kasturi L, Olechnowicz J, et al. Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. *Proc Natl Acad Sci USA.* 1998;95:8698-8702.
17. Kane MF, Loda M, Gaida GM, et al. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective humor tumor cell lines. *Cancer Res.* 1997;57:808-811.
18. Hendriks YM, Wagner A, Morreau H, et al. Cancer risk in hereditary nonpolyposis colorectal cancer due to MSH6 mutations: impact on counseling and surveillance. *Gastroenterology.* 2004;127:17-25.
19. Haugen AC, Goel A, Yamada K, et al. Genetic instability caused by loss of MutS homologue 3 in human colorectal cancer. *Cancer Res.* 2008;68:8465-8472.
20. Jung B, Doctolero RT, Tajima A, et al. Loss of activin receptor 2 protein expression in microsatellite unstable colon cancers. *Gastroenterology.* 2004;126:654-659.
21. Carethers JM, Hawn MT, Greenson JK, et al. Prognostic significance of allelic lost at chromosome 18q21 for stage II colorectal cancer. *Gastroenterology.* 1998;114:1188-1195.
22. Carethers JM, Smith EJ, Behling CA, et al. Use of 5-fluorouracil and survival in patients with microsatellite unstable colorectal cancer. *Gastroenterology.* 2004;126:394-401.
23. Huang SC, Chen CR, Lavine JE, et al. Genetic heterogeneity in familial juvenile polyposis. *Cancer Res.* 2000;60:6882-6885.
24. Boland CR, Thibodeau SN, Hamilton SR, et al. 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.
25. Huang SC, Lavine JL, Boland PS, et al. Germline characteristics of early age onset HNPCC. *J Pediatr.* 2001;138:629-635.
26. Chang DK, Ricciardiello L, Goel A, et al. Steady-state regulation of the human DNA mismatch repair system. *J Biol Chem.* 2000;275:29178.
27. Zigman AF, Lavine JE, Jones MC, et al. Localization of the Bannayan-Riley-Ruvalcaba syndrome gene to chromosome 10q23. *Gastroenterology.* 1997;113:1433-1437.
28. Kurland JE, Beck SE, Solomon CJ, et al. Cyclooxygenase-2 expression in polyps from a patient with juvenile polyposis syndrome with mutant BMPR1A. *J Pediatr Gastroenterol Nutr.* 2007;44:318-325.
29. Parsons R, Li GM, Longley M, et al. Mismatch repair deficiency in phenotypically normal human cells. *Science.* 1995; 268:1336-1338.
30. Halvarsson B, Lindblow A, Johansson L, et al. Loss of mismatch repair protein immunostaining in colorectal adenomas from patients with hereditary nonpolyposis colorectal cancer. *Mod Pathol.* 2005;18:1095-1101.
31. Harrington JM, Kolodner RD. Saccharomyces cerevisiae Msh2-Msh3 acts in repair of base-base mispairs. *Mol Cell Biol.* 2007;27:6546-6554.
32. Chi SG, Kim HJ, Park BJ, et al. Mutational abrogation of the PTEN/MMAC1 gene in gastrointestinal polyps in patients with Cowden disease. *Gastroenterology.* 1998;115:1084-1089.
33. Guanti G, Resta N, Simone C, et al. Involvement of PTEN mutations in the genetic pathways of colorectal cancerogenesis. *Hum Mol Genet.* 2000;9:283-287.
34. Boland CR, Koi M, Chang DK, et al. The biochemical basis of microsatellite instability and abnormal immunohistochemistry and clinical behavior in Lynch syndrome: from bench to bedside. *Fam Cancer.* 2008;7:41-52.
35. Chang CL, Marra G, Chauhan DP, et al. Oxidative stress inactivates the DNA mismatch repair system. *Am J Physiol Cell Physiol.* 2002;283:C148-C154.
36. Chang D, Goel A, Ricciardiello L, et al. Effect of H2O2 on cell cycle and survival in DNA mismatch repair-deficient and -proficient cell lines. *Cancer Lett.* 2003;195:243-251.
37. Lee SY, Chung H, Deveraj B, et al. Microsatellite alterations at selected tetranucleotide repeats are associated with morphologies of colorectal neoplasias. *Gastroenterology.* In press.