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Frequency of Deletions of *EPCAM* (*TACSTD1*) in *MSH2*-Associated Lynch Syndrome Cases

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Lynch syndrome is an autosomal dominant cancer predisposition syndrome characterized by loss of function of DNA mismatch repair enzyme MLH1, MSH2, MSH6, or PMS2. Mutations in *MLH1* and *MSH2* account for ~80% of the inherited cases. However, in up to 20% of cases suspected of having a germline mutation in *MSH2* due to loss of MSH2 expression, a germline mutation is not identified. Recent studies have shown that some Lynch syndrome cases are due to 3′ *EPCAM/TACSTD1* deletions that subsequently lead to *MSH2* promoter hypermethylation. In this study, we examined the frequency of this novel mechanism for *MSH2* inactivation in cases recruited through the Colon Cancer Family Registry and from the Mayo Clinic Molecular Diagnostics Laboratory. From the combined cohort, 58 cases were selected in which immunohistochemical staining suggested a mutation in *MSH2* or *MSH6*, but no mutations were identified on follow-up testing. Of these 58 cases, 11 demonstrated a deletion of *EPCAM/TACSTD1*. Of cases with a deletion, the methylation status of the *MSH2* promoter was confirmed in tumor tissue using methylation-sensitive PCR primers. One case showed *MSH2* promoter hypermethylation in the absence of a detectable *EPCAM/TACSTD1* deletion. These results indicate that approximately 20% to 25% of cases suspected of having a mutation in *MSH2* but in which a germline mutation is not detected, can be accounted for by germline deletions in *EPCAM/TACSTD1*. These data also suggest the presence of other alterations leading to *MSH2* promoter hypermethylation. *(J Mol Diagn 2011, 13:93–99; DOI: 10.1016/j.jmoldx.2010.11.011)*

Lynch syndrome is an autosomal dominant predisposition syndrome in which patients have a propensity to develop colorectal adenocarcinoma, endometrial carcinoma, sebaceous neoplasms, upper urinary tract urothelial carcinomas, central nervous system neoplasms, and ovarian and hepatobiliary neoplasms.[1–4] The underlying genetic basis for this syndrome is the presence of a mutation in one of the DNA mismatch repair genes *MLH1, MSH2, MSH6* or *PMS2*.[5–10] The defining phenotype of tumors from these patients is the presence of tumor microsatellite instability[11–13] and loss of protein expression of the affected enzyme in the tumor nuclei as detected by immunohistochemical staining.[14–16] The spectrum of mu-

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tations in these genes includes missense, nonsense, splice site alterations, insertion/deletions, and large gene rearrangements. Mutations in *MLH1* and *MSH2* are the most common and account for ~80% of the inherited mismatch repair cases.[17–19] Of the cases with loss of MSH2 immunohistochemical staining, approximately two-thirds of identifiable mutations in *MSH2* are point mutations or small insertions and deletions, whereas the remaining one-third are large gene rearrangements and deletions.[20,21] However, mutations in *MSH2* are not identified in up to 20% of the cases expected to have such an alteration.

Recently, germline deletions involving the 3′ end of *EPCAM* (also known as *TACSTD1*), located approximately 16-kb upstream of *MSH2*, have been shown to result in hypermethylation of the *MSH2* promoter region and subsequent loss of MSH2 expression from the affected allele.[22] In this study, we determined the frequency of this novel mechanism for *MSH2* inactivation and correlated results of multiplex ligation-dependent probe amplification (MLPA)[23,24] testing with results of the *MSH2* promoter hypermethylation test. Although specific endpoints of the deletions were not determined, the loss of material in the 3′ untranslated region is similar to the different size deletions previously reported.[22]

Materials and Methods

Case Selection

Colorectal cancer cases were selected from the Colon Cancer Family Registry (Colon CFR), a National Cancer Institute–supported consortium established in l997 to create a multinational comprehensive collaborative infrastructure for interdisciplinary studies in the genetic epidemiology of colorectal cancer. Detailed information about the Colon CFR, including method of ascertainment and molecular testing, can be found at http://epi.grants.cancer.gov/CFR/ (last accessed: March 19, 2010) as well as being described in detail by Newcomb et al[25] and Poynter et al.[26] Six Colon CFR sites used to identify eligible cases were: Seattle Familial Colorectal Cancer Registry, Hawaii Family Registry of Colon Cancer, Ontario Familial Colorectal Cancer Registry, Australasian Colorectal Cancer Family Study, University of Southern California Consortium, and Mayo Colorectal Cancer Family Registry. These sites use various strategies for recruitment such that the entire spectrum of colorectal cancer risk is represented, including participants recruited both from population-based sources and clinic-based sources. Inclusion criteria for the current study included: i) evidence of defective DNA mismatch repair by the loss of normal immunohistochemical staining of MSH2/MSH6 in tumor cell nuclei from diagnostic tumor samples; ii) the presence of the microsatellite instability-high tumor phenotype (if assessed); iii) no identifiable *MSH2* or *MSH6* mutation by sequencing and/or large gene rearrangement assays at the time of testing; and iv) sufficient clinical information and tumor material available for testing. Of 5927 cases in the Colon CFR database, 37 cases met these criteria. Approval from the institutional review boards of each participating CFR site was obtained. Colon CFR participants all provided informed consent.

The clinical Microsatellite instability/Lynch syndrome database of the Molecular Genetics Laboratory at Mayo Clinic containing 5598 cases (all tumors that had been clinically tested for Lynch syndrome, predominantly but not exclusively colorectal cancer was also used to select cases for this study. This dataset (case series) was collected from 2001 to 2008 and contains Lynch syndrome screening results from microsatellite instability and immunohistochemical tests, demographic information provided to the laboratory, and follow-up testing results including germline analysis as provided by the ordering physician or as clinically performed at Mayo Clinic. The same inclusion and exclusion criteria as described for the Colon CFR were applied to the cases from the Mayo database. Of the 5598 cases, 344 had evidence of abnormal MSH2 protein expression, with 100 having evidence of at least partial follow-up germline testing. In 21 of these cases, no alterations were identified. Mayo Clinic Institutional Review Board approval was obtained.

Microsatellite Instability Immunohistochemistry and Germline Testing

Microsatellite instability data from the Colon CFR and from Mayo Clinic were determined using the same panel of 10 microsatellite markers using standard procedures, techniques, and classification as described previously.[16,25] For some of the more recent Mayo Clinic cases, MSI testing was performed with the commercially available Promega MSI kit (Promega Corp., Madison, WI). Immunohistochemistry was performed according to previously described and standard protocols.[16,27] Germline

Table 1. Summary of MLPA and *MSH2* Promoter Hypermethylation Results

Case source (n)	# Tested by MLPA	Deletion identified	# Tested by methylation	*MSH2* promoter hypermethylation final call		
				Pos	Neg	No amp
Colon CFR (37)	36	5	35	5*	19	11†
Mayo Clinic (21)	21	6	15	6	9	0
Total (58)	57	11	50	11	28	11

MLPA, multiplex ligation-dependent probe amplification; Pos, positive; Neg, negative; amp, amplification; Colon CFR, Colon Cancer Family Registry.
*One case with *MSH2* promoter hypermethylation did not show an *EPCAM/TACSTD1* deletion.
†One case with a deletion failed for the *MSH2* promoter hypermethylation assay.

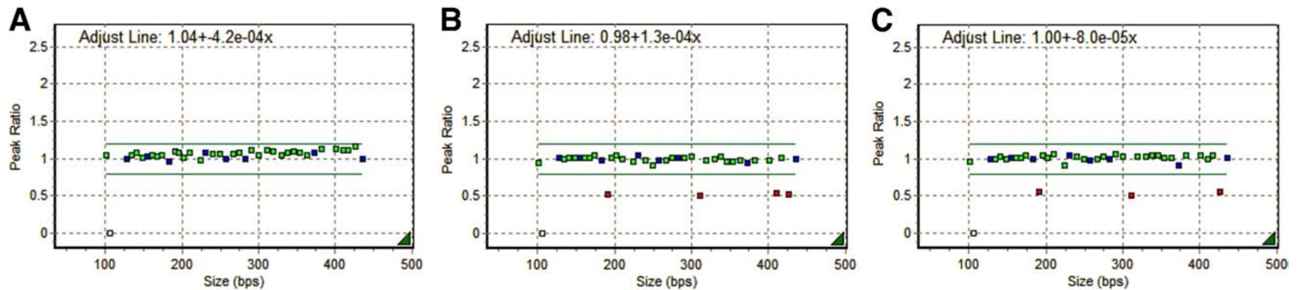

Figure 1. Analysis of *EPCAM/TACSTD1* for large deletions and duplications by MLPA, two normal copies of each probe (green squares) and control probes (blue squares) are represented by a peak ratio of one. Loss of one allele at the probe site results in a decrease in the peak ratio to 0.5 and is represented by a red square. **A:** Shows a normal result, with all *EPCAM/TACSTD1* probes showing normal dosage. **B:** Shows a deletion that includes exons 8 and 9 of *EPCAM/TACSTD1* and two probes located downstream of the gene including a probe 3 kb away. **C:** Shows a deletion that includes exons 8 and 9 only.

analysis was performed using a variety of methods including screening by denaturing high-performance liquid chromatography followed by sequencing of abnormal bands, direct Sanger sequencing, and Southern blot analysis or MLPA for large genomic deletions and insertions.[24,25,28,29]

MSH2 *Promoter Hypermethylation Assay*

Ten-micron-thick sections were cut from formalin-fixed, paraffin-embedded tissue from the selected cases. Normal and tumor tissue was macrodissected using a hematoxylin and eosin template. The slides were all tested at a single institution (Mayo Clinic). Bisulfite treatment was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) with modification of the method. Briefly, selected tissue was scraped into digestion buffer with Proteinase K and digested overnight at 50°C. This lysate was spun down, 5 μl of M-Dilution buffer and 5 μl of water were added and incubated at 37°C for 15 minutes. CT Conversion Reagent (100 μl) was added and incubated overnight at 50°C. The next day, 400 μl of M-Binding Buffer was added to Zymo-Spin I columns, followed by the sample. The columns were washed with 200 μl of M-Wash Buffer; 200 μl of M-Desulphonation Buffer was added and incubated at room temperature for 15 minutes, followed by two washes with 200 μl of M-Wash Buffer and elution of DNA into 10 μl of M-Elution Buffer.

The DNA methylation status of the *MSH2* promoter was tested using methylation-specific PCR primers based on the ref Gene-NM-000251 by Ligtenberg et al[22] These primers target two regions of the *MSH2* promoter (regions 1 and 3) and result in expected fragment sizes of 145 and 137 bp for the unmethylated and methylated amplicons of region 1, and 216 and 209 bp of region 3, respectively.[22] One microliter of bisulfite-treated DNA was used in each PCR reaction. Cycling conditions were modified as follows. For methylated and unmethylated primers for region 1 and the unmethylated primer for region 3, an initial denaturation step of 94°C for 10 minutes followed by 35 cycles of 92°C for 45 seconds, 65°C to 62°C for 45 seconds an extension of 72°C for 30 seconds, and a final extension of 72°C for 10 minutes. The region 3 methylated primers were run for 40 cycles with a similar thermocycler program except the annealing temperature was 62°C. Fragment analysis was performed on an ABI 3130X automated sequencer (Applied Biosystems, Foster City, CA) using standard methods and internal size marker ladder and instrument protocol 400-500bp-POP7-D.

Scoring of the *MSH2* promoter hypermethylation assay was performed as follows. The interpretation cutoff for fluorescent intensity was set at 500 relative fluorescent units for the untreated DNA tube, otherwise the sample was considered failed for that region. A sample was equivocal if the untreated DNA signal was >500 but the bisulfite-treated signal was greater than 0 and less than 500. Cases in which both primers gave a signal >500 were called positive for *MSH2* hypermethylation for that promoter region. Both regions 1 and 3 were taken into account for the final DNA methylation call. Only one positive region was needed for a final positive call. Equivocal and negative calls were conservatively called negative, and if one of the two regions failed, the score from the other region was used.

EPCAM/TACSTD1 *Deletion*

EPCAM/TACSTD1 deletion analysis was performed on DNA extracted from peripheral blood using a commercially available MLPA kit (P072 version 6; MRC Holland, Amsterdam, the Netherlands) at one institution (Mayo Clinic).[23,24] This kit contains oligonucleotide probes targeting *EPCAM/TACSTD1* exons 3, 8, 9, and two probes in the intervening region between *EPCAM/TACSTD1* and *MSH2*: one 3 kb downstream and one 2.5 kb upstream from the *MSH2* gene. Further delineation of the deletion breakpoints was not performed.

Results

Of the 11,525 cases from the Colon CFR and Mayo Clinic's clinical database, 37 and 21 cases, respectively, were identified in which loss of MSH2/MSH6 immunohistochemical stains could not be explained by follow-up germline testing. Of these 58 cases, 11 (19%) demonstrated a deletion within *EPCAM/TACSTD1*: 6 of 21 (29%) Mayo Clinic cases and 5 of 37 (14%) Colon CFR cases (Table 1). Examples of typical positive and negative cases are shown in Figure 1. All 11 deletions encompassed *EPCAM/TACSTD1* exons 8 and 9 (del 8/9), whereas five cases showed larger deletions extending at least 3 kb downstream of the *EPCAM/TACSTD1* coding region (del

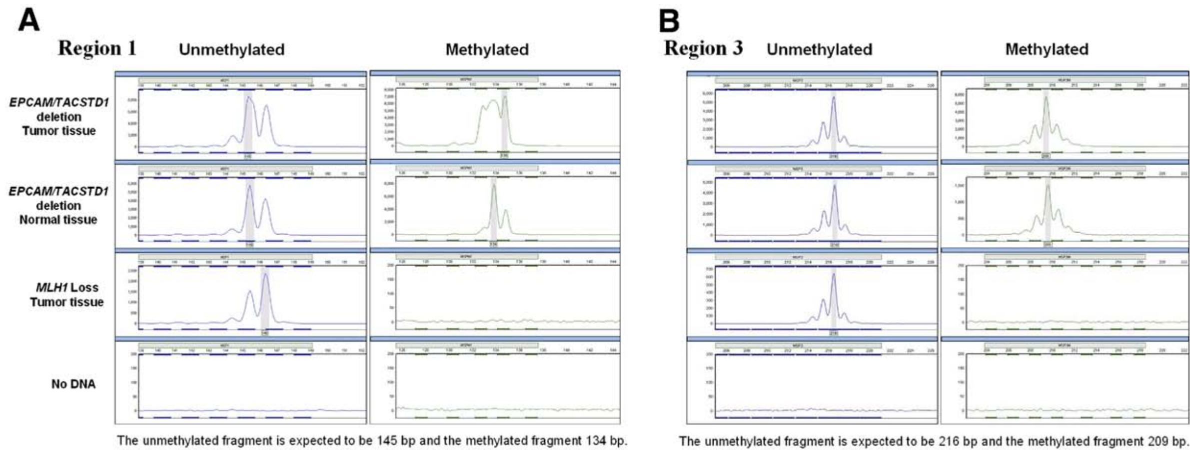

Figure 2. Results of *MSH2* hypermethylation test by methylation-specific PCR and fragment length analysis. **A:** Shows the results of the ABI-based fragment analysis for methylation-specific primers in region 1 of the *MSH2* promoter. *MSH2* hypermethylation can be seen in tumor and normal tissue in a case with a *EPCAM/TACSTD1* deletion. In contrast, the negative control case (loss of MLH1 expression) does not show *MSH2* methylation. **B:** Shows similar data for region 3 of the *MSH2* promoter.

8/9/3kb). None of the deletions involved exon 3 of *EPCAM/TACSTD1* or the coding region of the *MSH2* gene.

Hypermethylation of the *MSH2* promoter region was also tested in tissue samples from 50 of the 58 cases in which tissue was available (example shown in Figure 2). Eleven tissue samples (22%) demonstrated *MSH2* promoter hypermethylation. Of the eleven cases with an *EPCAM/TACSTD1* deletion, 10 demonstrated the presence of *MSH2* promoter hypermethylation, including all six cases from the Mayo Clinic group and four of the Colon CFR cases. One case with a deletion failed the *MSH2* hypermethylation assay due to poor amplification. Finally, there was one case within the Colon CFR cohort that showed *MSH2* promoter hypermethylation without a detectable *EPCAM/TACSTD1* deletion.

We compared demographic information between individuals in the study in which an *EPCAM/TACSTD1* deletion/*MSH2* promoter hypermethylation ($N = 12$) was identified with those in which such a deletion was not identified ($N = 46$) (Table 2). The average age of diagnosis for those cases with a deletion was 53 years compared to 51 years (age at the time tumor was being tested) for those in the study without these abnormalities. The sex distribution (male/female) was approximately 6:5 and 1:1 for the cases with and without a deletion.

Since the putative mechanism of *MSH2* gene inactivation involves the expression of another gene, *EPCAM/TACSTD1*, the question of whether or not individuals and families with this alteration would be affected by the same spectrum of tumors as typically seen in Lynch syndrome was raised. First, we looked at tumors that were initially tested for Lynch syndrome, the primary site was available in 50 cases. Thirty-two tumors sent for testing were colorectal primaries, 12 (38%) of which had evidence of *EPCAM/TACSTD1* deletion or *MSH2* promoter hypermethylation. None of the 18 non-colon tumors sent for testing were found to have an *EPCAM/TACSTD1* abnormality (Table 2). Second, to further investigate the spectrum of cancers in those families demonstrating a deletion in *EPCAM/TACSTD1*, additional personal and family history was examined from the two respective databases (available in 10 of 12 cases). For these 10 available families, 95 neoplasms were recorded among 83 individuals. Of the 95 neoplasms, there were 62 Lynch-related colorectal lesions (adenocarcinomas and adenomatous polyps), as well as 10 non-colon malignancies (stomach, uterine, ovarian, sebaceous, and pancreatic cancer). Finally, 23 tumors not typically associated with Lynch syndrome were observed (Table 3). Testing for the presence of defective mismatch repair (microsatellite instability and/or immunohistochemistry) was not performed on any of these additional tumors either from the proband or from

Table 2. Summary of Demographic and Tumor Characteristics in Cohort

	EPCAM/TACSTD1 deletion or *MSH2* hypermethylation			
	Present		Absent	
Sample source	Mayo Clinic $n = 6$	Colon CFR $n = 6$	Mayo Clinic $n = 15$	Colon CFR $n = 23$
Average age (years)	54	52	52	51
Sex ratio	3 M/3 F	4 M/2 F	6 M/9 F	13 M/10 F
Tumor tested in proband	6 Colorectal	6 Colorectal	8 Colorectal 4 Sebaceous 3 Uterine	22 Colorectal 1 Breast
Meet revised Bethesda Guidelines	5 of 5	5 of 6	10 of 13	NA

Colon CFR, Colon Cancer Family Registry; M, male; F, female.

Table 3. Demographics and Characteristics of Cases with *EPCAM/TACSTD1* Deletion /*MSH2* Promoter Hypermethylation

Sex	Age*	Number of probes deleted	*MSH2* hypermethylation	Meets revised Bethesda Guidelines	Tumor tested in proband	Other tumors in proband	Tumors in family members (<50 years)	Tumors in family members (>50 years or age not available)
M	52	4	Identified	Yes	CRC	—	—	CRC and stomach cancer
M	49	3	Identified	Yes	CRC	CRC	Melanoma, CRC ×2	CRC ×3, breast, bone, stomach, prostate and upper limb cancer†
M	58	0	Identified	Yes	CRC	—	—	Pancreatic and uterine cancer
F	68	3	Identified	Yes	CRC	—	CRC	CRC ×6, melanoma
F	35	3	Identified	Yes	CRC	—	NA	NA
M	NA	4	Identified	Yes	CRC	CRC ×2	CRC	ovarian cancer
M	59	3	Identified	Cannot determine	CRC	NA	NA	NA
F	47	3	Identified	Yes	CRC	—	CRC ×4	—
F	40	4	Identified	Yes	CRC	—	CRC ×3, extra-colonic tumor,† stomach, testicular, lung and gynecologic cancers	CRC, BCC, breast, prostate ×2, and liver cancer
M	69	4	Identified	Yes	CRC	CRC	CRC ×3 and pancreatic cancer	CRC ×3, and stomach cancer
F	60	4	Identified	Yes	CRC	Sebaceous carcinoma, bladder papillary urothelial carcinoma, SCC	CRC ×4, and colonic polyps	CRC ×2, and breast cancer ×2
M	51	3	Identified	Yes	CRC	CRC	Sarcoma†	CRC ×7, colonic polyps ×3, prostate cancer ×2, breast and renal cancer

M, male; CRC, colorectal adenocarcinoma; F, female; NA, not available; BCC, basal cell carcinoma; SCC, squamous cell carcinoma; —, none.
*Age at diagnosis of the sample being tested as reported at the time of testing.
†Histologic type and tumor location not specified.

family members. Third, we also looked at whether or not the probands would meet revised Bethesda Guidelines.[30] Based on available clinical and family history, 11 of the 12 cases met revised Bethesda Guidelines.

Discussion

In this study, we have demonstrated that deletions in the *EPCAM/TACSTD1* gene account for a significant fraction (approximately 20%) of cases suspected of having an *MSH2* germline mutation but in which a mutation was not identified using standard laboratory-based approaches. This result is consistent with previously published reports showing that this alteration has been observed in approximately 19% to 27% of cases with unidentified germline abnormalities.[31,32]

The presence of the *EPCAM/TACSTD1* gene deletion correlates with DNA hypermethylation of the *MSH2* promoter. Heritable DNA methylation abnormalities have been identified in two Lynch syndrome–associated genes, *MLH1*[33–40] and *MSH2*.[22,31,32,41,42] Heritable *MLH1* promoter hypermethylation appears to be a relatively rare event, and the mechanism for this alteration has not yet been determined. Evidence presented in the literature[22] proposes that the hypermethylation of the *MSH2* promoter region results from the production of an abnormal *EPCAM/TACSTD1* RNA; specifically, a deletion of the *EPCAM/TACSTD1* transcriptional termination signal results in a fusion transcript that includes at least part of *MSH2*. This abnormal transcript has been postulated to mediate the DNA hypermethylation of the *MSH2* promoter in *cis*.[22,31] This transcription-mediated inactivation of gene expression via epigenetic regulation has been previously reported in the α-globin gene locus.[43,44]

In this study, the *MSH2* methylation-specific PCR confirmed the association between the upstream *EPCAM/TACSTD1* deletion and *MSH2* promoter hypermethylation in cases with available DNA. In addition, the *MSH2* promoter hypermethylation assay can also identify rare cases in which MLPA for the *EPCAM/TACSTD1* 3′ region is not deleted. Indeed, one example of such a case was identified in the Colon CFR cohort in which *MSH2* methylation was detected but a deletion was not found by MLPA. The mechanism for the *MSH2* promoter hypermethylation in this case has not yet been elucidated. To rule out the possibility of specimen misidentification, DNA from both peripheral blood and tissue were genotyped, which showed that the two samples did indeed belong to the same individual. One explanation for the discordant results could be that this individual is tissue mosaic for the deletion—MLPA was not performed on DNA from the formalin-fixed, paraffin-embedded tissue. However, a more likely explanation for this discordant result is that this proband has either a small deletion, not detectable by the current MLPA probes, or a point mutation in the transcription termination signal of *EPCAM/TACSTD1* resulting in the same RNA read through that is currently thought to mediate the abnormal promoter hypermethylation of *MSH2*. This case demonstrates that the currently available MLPA test may not be 100% sensitive for *EPCAM/TACSTD1*-mediated events.

The resulting clinical phenotype of an *EPCAM/TACSTD1* deletion is dependent on the co-expression of EPCAM/TACSTD1 and MSH2. Thus, we questioned whether the tumor spectrum in cases with a deletion would be different from those without *EPCAM/TACSTD1* involvement. *EPCAM/TACSTD1* and *MSH2* have somewhat different expression patterns in normal and tumor tissues. The expression pattern of EPCAM/TACSTD1 in normal tissues is primarily associated with epithelial tissues. MSH2 is

more ubiquitous in normal tissue, although the levels of protein expression vary in different tissue types (GeneCards ID GC02P047572 at *http://www.genecards.org*, last accessed: April 6, 2010). High levels of expression have been associated with organs typically at risk in Lynch syndrome (colon, small bowel, stomach, endometrium, ovary, and transitional epithelium of the upper urinary tract). However, MSH2 expression can be seen in a range of other normal tissues including squamous epithelium, lung bronchi, pancreas, brain, testis, thyroid, prostate, and breast.[45]

Does the overlap of expression patterns for these two genes affect the tumor spectrum for this subset of Lynch patients? Based on the initial tumor sites tested (Table 2), and on previous work,[31] the data suggested that patients with a *EPCAM/TACSTD1* deletion may be over-represented with colorectal cancer only. Given the bias in the selection of tumors being tested in this albeit large cohort, additional personal and family history data were evaluated to determine what other tumors had occurred in the probands and their family members. On the basis of a review of available family history from the Mayo Clinic and Colon CFR databases, 95 tumors were reported among 83 individuals. The majority of tumors reported are colorectal. One family appears to be affected with colon cancer only, and several other families are predominantly affected with CRC, with only one or two non-colon tumors. However, there were four families that showed a range of tumors, having at least three non-colon tumors. This observation suggests that patients with an *EPCAM/TACSTD1* deletion may be at an increased risk of malignancies other than colon cancer.

There are a number of limitations regarding the tumor data. First, slides and/or pathology reports were not available for review for other tumors in the probands or for tumors reported in their families. In some cases, it is not clear whether some of the tumors reported represent second primaries, treatment-related tumors, or metastatic tumors. Some cancers (both Lynch and non-Lynch associated) occurred at older ages and may represent sporadic cancers in members of the family. Second, because tumor tissue from other Lynch and non-typical Lynch tumors was not available to us for additional studies, we were not able to test for microsatellite instability or expression of MSH2 protein by immunohistochemistry. Thus, we cannot determine whether these non-typical Lynch tumors are indeed associated with the germline defect or simply sporadic tumors within these families. Finally, the small number of cases identified with *EPCAM/TACSTD1* deletions (and *MSH2* promoter hypermethylation) precludes any definitive conclusion from being drawn regarding the spectrum of tumors in these patients. Nevertheless, the fact that nearly a quarter of tumors in these families were not typical Lynch syndrome tumors raises the possibility that affected individuals may be predisposed to a broader distribution of tumors and warrants additional study.

In summary, this study provides additional information regarding the frequency and spectrum of *EPCAM/TACSTD1* deletions and *MSH2* promoter hypermethylation in Lynch syndrome. This mechanism accounted for ~20% of cases in which colorectal cancer had loss of expression of MSH2/MSH6 but no previously identified germline mutation. Overall, this mechanism would account for approximately 5% of Lynch syndrome cases with abnormal MSH2/MSH6 expression. The presence of one discordant case in which *MSH2* promoter hypermethylation was detected in the tumor but a deletion was not identified by the current MLPA panel suggests that other mutations and/or somatic events may occur in rare cases. Although not definitive, our data suggest that patients with an *EPCAM/TACSTD1* deletion are at risk for malignancies in a variety of tissue types.

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