

# The Gene Variant *MLH1*\**D132H* is Not Associated with an Increased Risk for Colorectal Adenomas in a Suburban United States Jewish Population

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**Abstract:** *Background:* *MLH1*\**D132H*, a variant of the mismatch repair gene *MLH1*, was reported linked to an increased risk for colorectal carcinoma in Israeli patients. We evaluated U.S. Jewish patients with adenomas for this gene variant and compared the results to that of three other minor variants.

*Methods:* DNA was screened for the *MLH1*\**D132H* variant using standard PCR followed by melting point analysis or by single-stranded conformation polymorphism.

*Results:* Of the 632 patients screened there were three carriers among patients with adenomas and one carrier among those patients with no lesions for an incidence of 0.63% and a relative risk of 0.81 (C.I.=0.08-7.85, p=1.00).

*Conclusion:* We did not detect a significant risk for colorectal adenoma in association with the *MLH1*\**D132H* variant. The personal, family history and germ line features of the carriers illustrate the complexity of minor genetic determinants.

## INTRODUCTION

*MLH1* is one of several genes important in DNA mismatch repair. Germ line mutations of this gene can be identified in many patients with the autosomal dominant hereditary cancer syndrome Hereditary Non-Polyposis Colorectal Cancer (HNPCC), or Lynch syndrome [1]. These patients have an increased risk for the development of colorectal lesions, both adenomas and carcinomas, with microsatellite instability (MSI) [2]. A variant *MLH1* allele, referred to as *MLH1*\**D132H*, was recently identified that attenuates, but does not completely eliminate production of the *MLH1* protein. Colorectal carcinomas occurring in individuals with this particular germ line mutation do not demonstrate microsatellite instability [3].

In one study, the heterozygous carrier state for the *MLH1*\**D132H* allele was estimated to confer almost a five-fold increase in colorectal cancer risk, with an overall allele frequency estimated at about 1.3% of colorectal cancer patients among a predominately Jewish Israeli population [3]. We have been evaluating patients of Jewish ancestry for several germ line mutations and were interested in assessing the frequency of the *MLH1*\**D132H* mutation in our patient population. In particular, we were interested in the frequency of this mutation among Jewish patients with documented colorectal adenomas, the precursors for many colorectal carcinomas. Our study population contained many more individuals with adenomas than with carcinomas, thereby providing us an

opportunity to assess for any impact of the *MLH1*\**D132H* mutation on these precursor lesions.

## MATERIALS AND METHODS

Our first cohort consisted of 394 patients of Northern European Jewish background who had a pathologically confirmed colorectal adenoma or carcinoma and were undergoing either their first or a follow up colonoscopy. The second series consisted of 238 individuals, also of Jewish background, who underwent a colonoscopy within the previous five years, regardless of findings. Ninety-one percent of all patients were asymptomatic at the time of colonoscopy and were undergoing either an initial screening (12%) or a follow-up surveillance colonoscopy (79%). Eight percent had symptoms leading to the evaluation and for one percent the indication was unclear. The institutional review board approved all studies and informed written consent was obtained from all participants. Patient participation was dependent upon verification of Jewish ancestry, patient consent and retrieval of stored paraffin-embedded colonic neoplastic tissue for analysis or donation of a blood sample. All information was entered into a stand-alone computer. Relative risks were calculated using Fisher's exact test.

## DNA Extraction and Purification

DNA was obtained from either peripheral blood or from formalin-fixed paraffin-embedded tissue sections. DNA from peripheral blood white cells was extracted using the Pure-gene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) and DNA from paraffin-embedded tissue was de-waxed, lysed and then extracted using the QIAamp Tissue Kit (Qiagen, Valencia, CA).

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### *MLH1*\**D132H* Melting Curve Analysis

Detection of the *MLH1*\**D132H* variant was performed by standard PCR followed by probe hybridization and melting analysis. PCR reactions were carried out in 25  $\mu$ l volumes using Applied Biosystems reagents (Roche Molecular Systems, Inc., Branchburg, NJ), 25 picomoles of each primer and a 2.0 mM MgCl<sub>2</sub> concentration. The primer set used was 5'-TTA TGG AAG TAG TGG AGA AA-3' and 5'-CCT GAA AAC TTA GAA GCA-3' and was ordered through Sigma Genosys ([www.sigma-genosys.com](http://www.sigma-genosys.com)). Reactions were performed in an ABI 9600 thermocycler (Applied Biosystems, Foster City, CA) under the following conditions: 5 minutes denaturation at 95°C, followed by 40 cycles of a 30 second denaturation at 94°C, 30 second annealing at 55°C, and a 60 second elongation at 72°C, with a final 10 minute extension at 72°C. Probes were added to the PCR tubes for melting analysis using the Light Cycler instrument (Roche Diagnostics Corporation, Indianapolis, IN). The anchor probe was 5'-LC640-CCT CCT AAA CCA TGT GCT GG-3' and the sensor probe is 5'-ACT CAC ATG GAA AAC TGA AAG-FL-3'. The anchor probe is 5'-labeled with LightCycler 640 and phosphorylated at the 3'-end. The sensor probe is labeled with fluorescein at the 3'-end. Anchor and sensor probes were ordered through Tib Molbiol, LLC, (Adelphi, NJ). Samples were loaded onto the LightCycler instrument and heated to 95°C for two minutes to denature. They were then cooled to 35°C, pausing at 55°C and 35°C for 2 minutes each, and melted from 35°C to 75°C. The D132H variant generated a melting peak at 59.5°C while the normal peak was at 50.0°C, the sensor probe being exactly complementary to the D132H variant (Fig. 1).

### *MLH1*\**D132H* SSCP Analysis

Single-stranded conformation polymorphism (SSCP) analysis was employed to screen for the *MLH1*\**D132H* variant for 90 samples unsuccessfully assayed with the LightCy-

cler. These samples contained too little DNA for the LightCycler but adequate amounts of DNA for SSCP, which has greater analytical sensitivity than the LightCycler. The primer set used was 5'-TTG GGA TTA GTA TCT ATC TCT CTA CTG G-3' (sense) and 5'-CTT GAT TGC CAG CAC ATG G-3' (antisense). The sense and antisense primers were 5'-labeled with different fluorescent tags and ordered through the Applied Biosystems Custom Oligo Synthesis Service ([OligosUS@appliedbiosystems.com](mailto:OligosUS@appliedbiosystems.com)). Reactions were performed in an ABI 9700 thermocycler (Applied Biosystems, Foster City, CA) under the following conditions: 5 minutes denaturation at 95°C, followed by 35 cycles of a 30 second denaturation at 94°C, 30 second annealing at 56°C, and a 60 second elongation at 72°C, with a final 30 minute extension at 72°C (Fig. 2).

After amplification the PCR products were mixed with formamide and a size marker and heated to 95°C for 5 minutes followed by flash cooling in ice water. The samples were analyzed on an ABI 3130 Genetic Analyzer with GeneMapper software (PE Applied Biosystems, Foster City, CA). The 3130 capillaries were loaded with a mixture that was 6.3% POP™ Conformational Analysis Polymer, 7% glycerol and 1X buffer and the samples underwent electrophoresis at 25°C.

### Sequencing Analysis

Samples that were positive for the *MLH1*\**D132H* variant by melting or by SSCP were sequenced to verify the point mutation. DyeDeoxy Terminator sequencing was performed with the BigDye™ Terminator Cycle Sequencing Kit on an ABI 9700 thermocycler (Applied Biosystems, Foster City, Ca). The PCR products were column-purified prior to the sequencing reaction using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) and were cleaned with AutoSeq G-50 spin columns (Amershan Pharmacia, Piscataway, NJ). Electrophoresis of the samples was performed

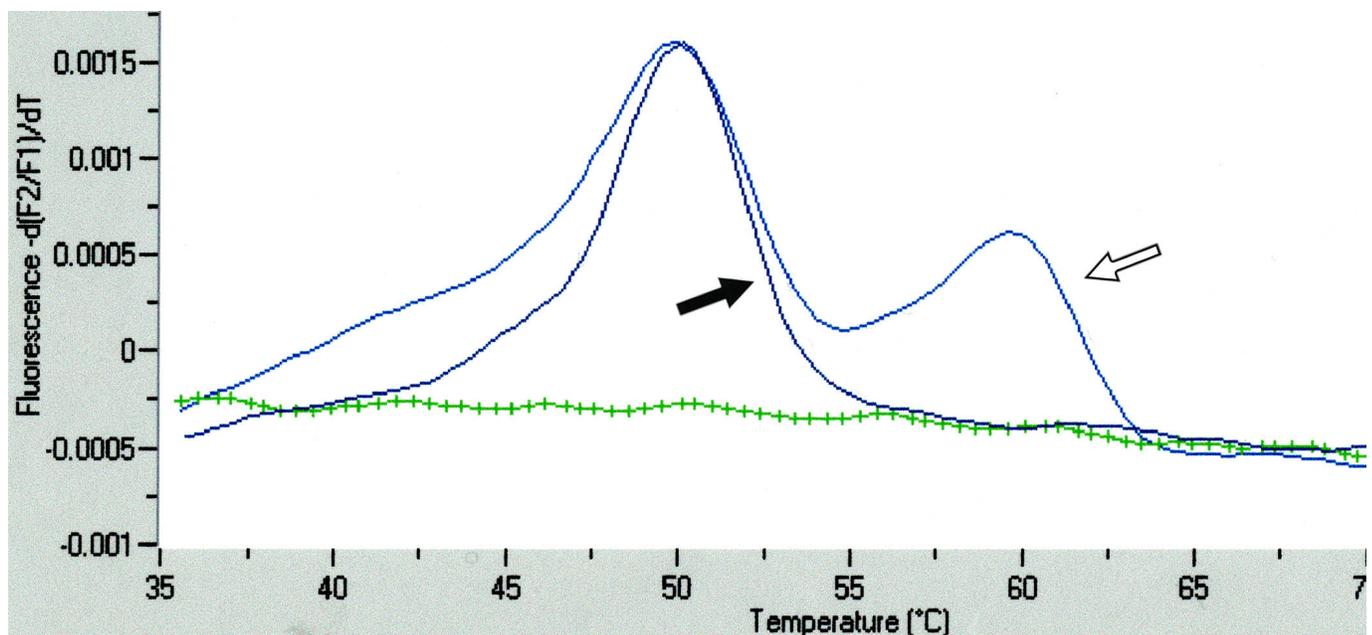
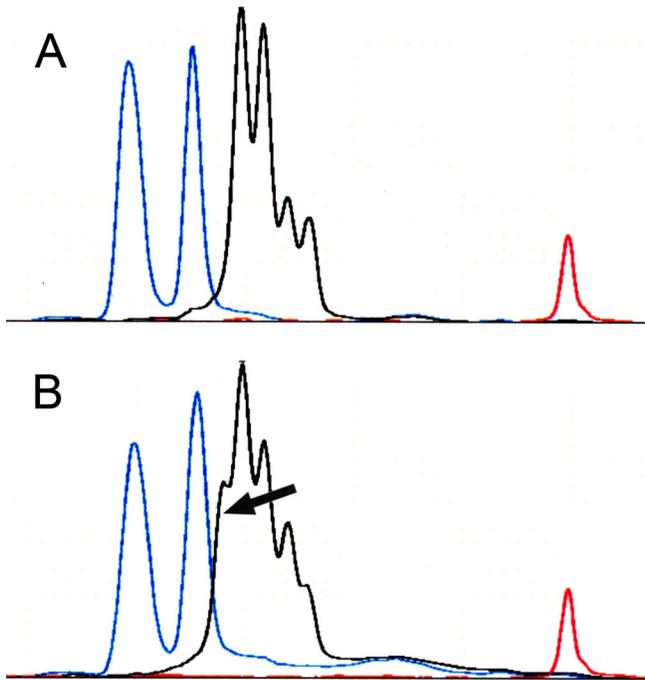


Fig. (1). Mutation screening using melting curve analysis. The solid arrow indicates a wild type sample with a melting peak at 50°C. The open arrow shows a sample that is heterozygous for the *MLH1*\**D132H* mutation with the normal 50°C melting peak and an additional melting peak (arrow) at 59.5°C corresponding to the mutant allele.

on an ABI 3130 Genetic Analyzer with POP-7™ polymer at 60°C and analyzed with Sequencing Analysis software. All analyses for the gene mutations APC\*I1307K, MSH2\* and Blooms<sup>Ash</sup> were performed by direct sequencing.



**Fig. (2).** Mutation screening using SSCP analysis. A. Wild type sample with the normal pattern. B. Heterozygous sample with the arrow indicating the extra peak reflecting the MLH1\*D132H mutation.

**RESULTS**

The two cohorts together consisted of 632 Jewish individuals, 376 males and 256 females for a M : F ratio of 1.47 : 1. The average age was 61.2 +/- 11.08 years. The patients can be subdivided into four groups: no colorectal lesions, adenomas only, cancer only, and both adenomas and cancer. The average age for each group at the time of first colonoscopy or first lesion was, no lesions: 57.2 +/- 10.2 years; adenomas only: 61.7 +/- 10.8 years; cancer only: 61.8 +/- 10.8 years; both adenomas and cancer: 66.8 +/- 12.8 years. The frequency of MLH1\*D132H for each group is shown in Table 1. For comparison, the frequencies of three other germ line mutations reported to be associated with colorectal neoplasm in Jewish patients are also included.

Four individuals were identified with the germ line mutation MLH1\*D132H, three males and one female, for an overall frequency of 0.63%. There were three carriers among the 474 patients with pathologically documented colorectal adenomas, for an frequency of 0.63%, and one carrier among those patients with no lesions, for an frequency of 0.78%. This results in a relative risk for the presence of MLH1\*D132H in a patient with an adenoma of 0.81 (C.I.=0.08-7).

The personal and family histories of the four carriers are informative. Proband # 1 underwent his first colonoscopy at age 66 years, with the removal of one tubular adenoma from the descending colon. A tubulovillous adenoma was removed three years later from the sigmoid colon. This patient was also a heterozygous carrier for the Adenomatous Polyposis Coli gene variant, APC\*I1307K. Proband # 2 underwent a left hemicolectomy for a carcinoma of the descending colon at age 42 years. He developed a tubular adenoma of the sigmoid two years later, but no further lesions over the next thirteen years. Proband # 3 had several sigmoidoscopies and three colonoscopies up to age 70 years. She had one tubular adenoma of the descending colon found at age 61 years. Proband # 4 had three colonoscopies between ages 66 and 75 years with only one hyperplastic polyp detected in the sigmoid colon (0.2 cm) on the first examination. Three of the four carriers have a first-degree relative with either colorectal carcinoma or adenoma, the fourth has a second-degree relative affected (Fig. 3).

The first-degree relatives of the four carriers have yet to be tested for the MLH1\* D132H variant. We obtained tissue blocks of three adenomas from one carrier and one carcinoma and one adenoma from another of the carriers. All five lesions were microsatellite stable when tested with the mononucleotide markers BAT26 and BAT25 and the dinucleotide marker DP1. All five of these lesions stained positively for MLH1 protein by immunohistochemistry (IHC). The tissue also stained positive for PMS2 protein, indicating no deficiency of this particular mismatch repair gene (IHC staining courtesy of Dr. Jeremy Jass, McGill University, Montreal, Canada).

**DISCUSSION**

The MLH1 gene has been mapped to chromosome 3p23-p21.3 [2]. The D132H variant is a change in the nucleotide base from guanine to cytosine at codon 415 (415G-C). This results in an amino acid substitution of histidine for aspartic acid. MLH proteins interact with the DNA-binding MSH proteins to catalyze mismatch repair in an ATPase-dependent manner. The D132H substitution attenuates, but does not eliminate, MLH1 ATPase activity [3].

**Table 1. Frequency of Susceptibility Mutations by Patient Groups**

Group	# People	M:F	MLH1*D132H		I1307K		MSH2*		Blooms <sup>Ash</sup>	
			#	(%)	#	(%)	#	(%)	#	(%)
No CR lesions	128	1.06	1	(0.8)	11	(8.6)	1	(0.8)	0	
Adenomas only	421	1.62	2	(0.5)	47	(11.2)	0		4	(0.9)
Cancer only	30	1.10	0		2	(6.7)	1	(3.3)	0	
Both adenomas and cancer	53	1.80	1	(1.8)	11	(20.8)	2	(3.8)	0	

\*MSH2\*1906G>C: 630 individuals assayed.  
 †624 individuals assayed.

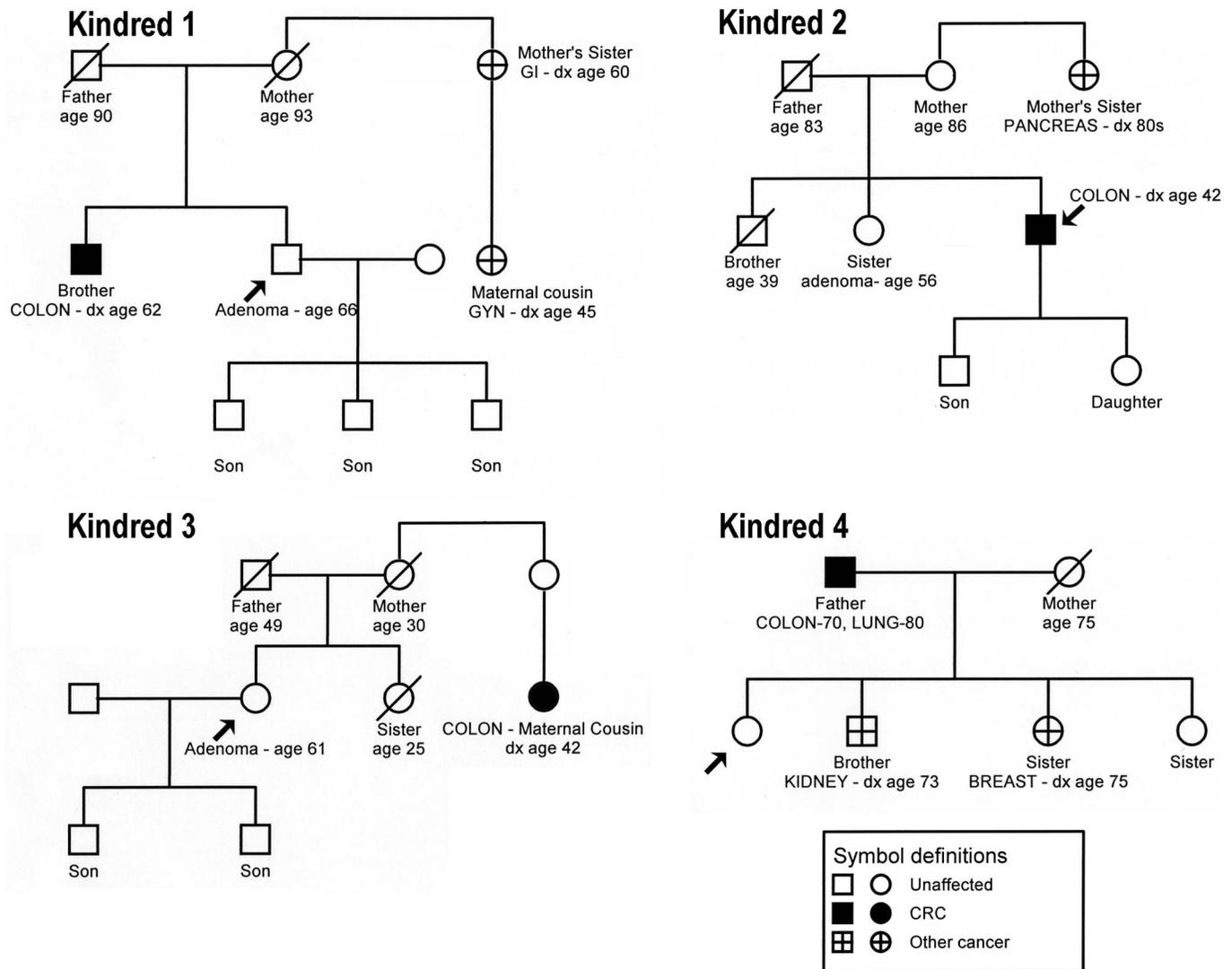


Fig. (3). Pedigrees of the carriers of the *MLH1*\**D132H* mutation.

Only two studies on the *MLH1*\**D132H* variant have been published to date [3, 4]. The first report detailed two series of colorectal cancer cases and matched controls from Israel. Although the majority of the patients were Jewish, the variant was also found in other groups that the authors indicate are self-described ethnicities: Muslim and Christian Arabs, Druze Christians and Bedouins. Twenty-one of 1231 (1.7%) cases carried the variant, while only 5 of 1353 (0.37%) controls, resulting in an odds ratio of 4.6 (C.I.=1.7-12.3). The second report details 629 cases of colorectal cancer and 515 cases of endometrial cancer from the United States. No patient was found to carry the *MLH1*\**D132H* variant, suggesting to the authors that the allele frequency is significantly lower in American cancer patients compared to Israeli cancer patients. Of note, however, only 2.7% of their colorectal cancer cases, or 17 individuals, were of Jewish ancestry. If the risk for carrying this gene variant is the same for the overall US as for the Israeli population, then 11 carriers would have been detected; but if this variant is found primarily in those of Jewish background, then less than one carrier would be expected ( $629 \times 0.027 \times 0.017 = 0.29$ ). Clearly, the frequency of the *MLH1*\**D132H* variant in the Jewish popu-

lation is low, and therefore the confidence interval around the relative risk, even for the Israeli study, is quite broad (1.7-12.3). Additional data may indicate the actual relative risk for colorectal cancer in Jewish patients is somewhat lower than 4.6. There are no published data on the frequency of this gene variant in the general healthy population, Jewish or non-Jewish.

All our patients had provided detailed personal histories and had undergone colonoscopies, thereby providing a full appreciation of the extent of their colorectal disease. However, even those individuals without lesions do not represent "controls" in terms of the entire general population. Some may have had symptoms or a relevant family history that led to the colonoscopy. Similarly, the controls in the one previous report of this mutation in Israelis used a roster of clinic patients as controls [3].

We have provided an estimate of the frequency of the *MLH1*\**D132H* mutation in a Jewish population with colorectal adenomas. Although the confidence interval of our estimate is wide, these results do suggest that the mutation is

less of a risk factor for the formation of adenomas than has been reported for carcinomas.

We also provide comparative data for three additional germ-line mutations that have been associated, in some series, with colorectal neoplasms. Some of these data were previously published [5]. The frequency of the *MLH1*\**D132H* mutation is similar to the frequencies we found for two other mutations, *Blm*<sup>Ash</sup> and *MSH2*\**A636P*; but less frequent than the *APC*\**I1307K* mutation [6-8]. One-eighth (12.6%) of our adenoma patients carry one of these four mutations. This is a sizable percentage, yet it does not preclude the possibility of other, currently unrecognized genetic mutations. Although a large study of Jewish patients would be required, it would be interesting to assess if these variants might act together to increase the risk for colorectal neoplasm in a polygenic manner, as suggested for breast cancer [9].

Of our four carriers, one individual had colon cancer at a young age, while another has had no colonic neoplasm through age 75 years. One individual who formed several adenomas also carries the *APC*\**I1307K* polymorphism. This phenotypic variability suggests the *MLH1*\**D132H* mutation may not be independently sufficient for neoplastic development. It also indicates that clinical findings may, on occasion, be attributed incorrectly to one low-penetrance variant when other, more etiological mutations may be unknown.

Adenomas are the precursor lesion for many colorectal carcinomas, and the colonoscopic removal of adenomas contributes to an overall reduction in colorectal carcinoma frequency [10]. As patients return for follow up surveillance colonoscopies, it is increasingly likely that adenomas, not carcinomas will be the primary lesions detected. It is therefore beneficial to ask whether known, or suggested, genetic predispositions to colorectal cancer apply equally to the development of colorectal adenomas. Our study, encompassing 474 patients with pathologically documented colorectal ade-

nomas, does not suggest a strong association of the *MLH1*\**D132H* variant with adenoma formation in a US Jewish population.

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