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Microsatellite Stable Colorectal Cancers in Clinically Suspected Hereditary Nonpolyposis Colorectal Cancer Patients without Vertical Transmission of Disease Are Unlikely to Be Caused by Biallelic Germline Mutations in *MYH*

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Microsatellite analysis and immunohistochemistry are commonly used initial screening tests for hereditary nonpolyposis colorectal cancer. However, tumors in roughly one-half of the patients fulfilling the Bethesda guidelines are microsatellite stable. In addition, normal mismatch repair protein expression in these tumors suggests that a defect in the mismatch repair system is unlikely. Because biallelic *MYH* mutations occur in patients with both high and low numbers of adenomas, we hypothesized that *MYH* is involved in the tumorigenesis of microsatellite stable colorectal cancers in patients without vertical transmission of disease and who fulfill the Bethesda guidelines. *MYH* was analyzed in 50 cancer patients and 116 healthy controls by complete genomic DNA sequencing. No biallelic germline mutations were identified. One patient was a heterozygous carrier for the p.G382D missense mutation, and another patient was a heterozygous carrier for the novel missense mutation p.Q484H. We identified six common variants, three in the coding region (p.V22M, p.Q324H, and p.S501F) and three in adjacent intronic regions (c.157+30A>G, c.462+35G>A, and c.1435–40G>C). In summary, biallelic germline mutations of *MYH* are unlikely to cause colorectal cancer in patients sharing clinical features with hereditary nonpolyposis colorectal cancer families without mismatch repair defect and therefore cannot fill the molecular diagnostic gap in this subgroup of Bethesda-positive patients. *(J Mol Diagn 2006, 8:178–182; DOI: 10.2353/jmoldx.2006.050119)*

Hereditary nonpolyposis colorectal cancer (HNPCC) is a common human cancer susceptibility syndrome that is caused by germline mutations in mismatch repair (MMR) genes in an autosomal dominant manner.[1]. Mutation carriers are at high risk for developing colorectal cancer as well as extracolonic carcinomas (particularly endometrial, small bowel, and ureter/renal pelvis carcinomas).[2,3] Tumors in HNPCC patients reveal a high level of microsatellite instability (MSI-H) as a fundamental indicator of MMR defects and constitute both a cause and an effect.[4] Immunohistochemistry can pinpoint the mutated gene through lost or reduced expression of the respective MMR protein.[1]

The newly defined Revised Bethesda Guidelines[5] have been established to provide a general recommendation regarding which tumors should be tested for microsatellite instability (MSI). Yet, roughly one-half of the patients fulfilling the clinical Bethesda criteria carry tumors with a microsatellite stable phenotype, and normal MMR protein expression in these cases implies that these tumors are unlikely to be caused by mutations in MMR genes.[2]

Several genes are good candidates to fill the molecular diagnostic gap, such as the *APC* gene, which is mutated in the germline of familial adenomatous polyposis patients, or *SMAD4*, which causes juvenile polyposis syndrome and is mutated in ~20% of sporadic colorectal cancers.[6] However, in the vast majority of microsatellite stable cases, the disease-causing genes are unknown.

MYH-associated polyposis (MAP) is a recently described syndrome (MIM 608456) with multiple colorectal adenomas and cancer, having an autosomal recessive mode of inheritance.[7–9] MAP is caused by germline mutations in the base excision repair gene *MYH*. The *MYH* gene is located on chromosome 1p and encodes a DNA glycosylase that excises adenines misincorporated into replicated DNA opposite 8-oxoguanine (for review, see Ref. [10]).

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Table 1. Newly Generated *MYH* PCR Primer Pairs

Exon	Sense primer	Antisense primer
3 to 5	5′-AAGGGGGTTAGTTGGGGGAAGC-3′	5′-CAAGGGTGAAGGTGGTAGAGGAAGC-3′
6 to 8	5′-TTTGGGGTGGGTGTAGAGAAGG-3′	5′-GCACAGAGGGGCCAAAGAGTTAG-3′
9 to 11	5′-CAGCCCACCCCCACTTTGT-3′	5′-GCTTTGGCCGGGTTCTGC-3′
12	5′-AGCCCCATTCCAGTTCTTCCTCTA-3′	5′-AGCTGCCGATTCCCTCCATTCTCT-3′
13	5′-AGGGCAGTGGCATGAGTAAC-3′	5′-GGCTATTCCGCTGCTCACTT-3′
14	5′-TTGGCTTTTGAGGCTATATCC-3′	5′-ACATGTAGGAAACACAAGGAAGTA-3′

Because both syndromes (HNPCC and MAP) share some clinical features, namely low number of polyps, young age of disease onset, and family history, we hypothesized that *MYH* is involved in tumorigenesis of colorectal cancers from patients suspected of HNPCC but without mismatch repair deficiency.

Materials and Methods

Subjects

We analyzed blood DNA samples from 50 consecutive patients from our HNPCC registry who fulfilled the following requirements. First, they fulfilled at least one criterion of the Revised Bethesda Guidelines: 1) colorectal cancer diagnosed under the age of 50 years ($n = 42$); 2) synchronous or metachronous colorectal or other HNPCC-associated tumors regardless of age ($n = 4$); 3) colorectal cancer with typical MSI-H histology diagnosed in a patient who is less than 60 years of age ($n = 0$); 4) colorectal cancer diagnosed with one or more first-degree relatives with HNPCC-related tumor, with one of the cancers diagnosed in a patient under age 50 years ($n = 3$); or 5) colorectal cancer diagnosed with two or more first- or second-degree relatives with HNPCC-related tumor, regardless of age ($n = 1$). Second, they had a family history showing no vertical transmission of disease (if there were affected relatives, then only siblings, consistent with an autosomal recessive inheritance). Third, tumors were microsatellite stable and showed normal protein expression for MSH2, MLH1, and MSH6 and also normal expression for PMS2 in 40 of the 50 tumors tested, indicating that mismatch repair deficiency is unlikely to be the cause of these tumors. We used 116 anonymous healthy blood donors as controls. All subjects were Caucasians from Germany.

Molecular Analysis

Analysis for microsatellite instability and immunohistochemical staining was performed as described previously.[11] Analysis of the *MYH* gene (Ensemble Gene ID ENSG00000132781; GenBank accession number NM_012222; EMBL accession number U63329) was performed on genomic DNA isolated from peripheral blood leukocytes obtained from all patients and controls using standard protocols.

We amplified from genomic DNA the 16 *MYH* exons, including flanking intronic regions, with primers described by Al Tassan et al[7] for exons 1, 2, 15, and 16. To amplify exons 3 to 14, we generated new primer pairs (Table 1). PCR products also included the last 33 nucleotides of intron 2 as described by Slupska et al[12] (EMBL U63329) and predicted to be a part of exon 3 (NM_012222, ENSG00000132781), to include both possible splice acceptor sites from intron 2. Additionally, our PCR products included the first 33 nucleotides of intron 14 as described by Slupska et al[12] (EMBL U63329) and predicted to be a part of exon 14 (ENSG00000132781) to comprise both possible splice donor sites from intron 14. In accordance with most published papers on *MYH,* we used numbering of nucleotides and codons as described in the original sequence by Slupska et al,[12] with a protein length of 535 amino acids.

Polymerase chain reactions contained 50 ng of DNA, 200 mmol/L of each dinucleotide, 1.2 to 3 mmol/L $MgCl_2$, 200 nmol/L of each primer, and 1 unit of Ampli*Taq* (PE Applied Biosystems, Weiterstadt, Germany) in a total volume of 25 μl. Conditions were 40 seconds at 94°C, 50 seconds at 56 to 67°C, and 1 minute at 72°C for 35 cycles with 5 minutes at 94°C before and 7 minutes at 72°C after cycling.

Amplified fragments were analyzed by direct DNA sequencing, applying the Thermo Sequenase Fluorescent Cycle Sequencing kit (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer's protocol. Sequencing primers were identical to PCR primers with additional Cy5-labeling, allowing sequence analysis on A.L.F.express devices (Amersham Pharmacia Biotech).

To assess the potential functional significance of the missense mutations, we applied sorting intolerant from tolerant amino acid substitutions (SIFT) analysis, which uses an evolutionary approach and is based on the assumption that important amino acids tend to be conserved across species. SIFT assigns a substitution probability from 0 to 1 for each possible amino acid change. Substitutions with probabilities <0.05 are considered as deleterious, whereas those ≥ 0.05 are inferred to be tolerated substitutions.[13]

Statistical Analysis

To evaluate deviations from Hardy-Weinberg equilibrium (HWE), observed and expected genotype frequencies were compared by an exact goodness-of-fit test separately for cases and controls. Allele frequencies between groups were compared performing an exact test. If there

Table 2. Adjusted OR and Exact 95% CI According to Detected *MYH* Polymorphism in HNPCC Patients ($n = 50$) versus Controls ($n = 116$)

			Controls		HNPCC				
Locus	Base Exchange	Type	*n*	%	*n*	%	OR	95% CI	*P* value*
Exon 2	c.64 G>A (p.V22M)	G|G	106	91.4	46	92			0.974
		G|A	10	8.6	3	6	0.868	(0.275, 3.143)	
		A|A	0	0	1	2	0.754	(0.076, 9.878)	
Exon 7	c.494 A>G p.(Y165C)	A|A	115	99.1	50	100			0.699
		A|G	1	0.9			1.000	(0.011, +Inf)	
		G|G							
Exon 12	c.972 G>C (p.Q324H)	G|G	69	59.5	39	78			0.014†
		G|C	41	35.3	11	22	2.430	(1.167, 5.530)	
		C|C	6	5.2	0	0	5.907	(1.361, 30.58)	
Exon 13	c.1145 G>A (p.G382D)	G|G	115	99.1	49	98			1.000
		G|A	1	0.9	1	2	0.429	(0.005, 34.13)	
		A|A							
Exon 15	c.1452 G>C (p.Q484H)	G|G	116	100	49	98			0.301
		G|C			1	2	1.000	(0.000, 16.81)	
		C|C							
Exon 16	c.1502 C>T (p.S501F)	C|C	113	97.4	47	94			0.510
		C|T	3	2.6	3	6	0.418	(0.054, 3.238)	
		T|T							
Intron 2	c.157+30 A>G	A|A	110	94.8	48	96			0.816
		A|G	4	3.4	2	4	1.509	(0.385, 11.75)	
		G|G	2	1.7	0	0	2.277	(0.148, 138.0)	
Intron 6	c.462+35 G>A	G|G	101	87.1	44	88			0.875
		G|A	13	11.2	6	12	1.212	(0.464, 3.695)	
		A|A	2	1.7	0	0	1.469	(0.215, 13.65)	
Intron 14	c.1435–40 G>C	G|G	102	87.9	44	88			1.000
		G|C	13	11.2	6	12	1.08	(0.388, 3.447)	
		C|C	1	0.9	0	0	1.166	(0.150, 11.88)	

*Given *P* values relate to Cochran-Armitage-Trend test and are calculated using StatXact-4.

†Because genotype distributions were significantly different between cases and controls, we analyzed a second sample of 63 patients. Comparison of this new cohort of 113 patients with 116 controls revealed no deviation from HWE and no significant difference in genotype frequencies (69 GG, 41 GC, and 6 CC genotypes in controls and 79 GG, 31 GC, and 3 CC genotypes in cases; $P = 0.105$).

CI, confidence interval; OR, odds ratio.

were deviations from Hardy-Weinberg equilibrium, a special asymptotic test according to Schaid et al[14] was performed. Odds ratios and exact 95% confidence intervals were calculated to compare genotype frequencies with the Cochrane-Armitage trend test. Statistical analyses were performed using SAS 9.1 (SAS Institute Inc., Cary, NC) and StatXact-4 (Cytel Software Corporation, Cambridge, MA). *P* values less than 0.05 were considered statistically significant.

Results

Genotype frequencies of all identified variants are listed in Table 2. We identified one patient with a previously unknown heterozygous missense mutation (c.1452G>C, p.Q484H) in exon 15, who had rectal cancer at the age of 42 and no family history of colorectal cancers. The mutation was not detected in 116 healthy controls. Another patient was a heterozygous carrier of the p.G382D missense mutation, which is known to be a pathogenic mutation associated with MAP.[7] This patient had a rectal carcinoma at the age of 48 without family history of colorectal carcinomas. We have also found this mutation in one of 332 control alleles. In addition, we identified the pathogenic mutation c.494A>G (p.Y165C)[7] in one control person but not in any patient.

Furthermore, we detected three previously reported coding-region variants in exon 2 (c.64G>A, p.V22M), in exon 12 (c.972G>C, p.Q324H), and in exon 16 (c.1502C>T, p.S501F) and three known intronic variants in intron 2 (c.157+30A>G), in intron 6 (c.462+35G>A), and in intron 14 (c.1435–40G>C), in patients as well as healthy controls. The polymorphisms in intron 6 (c.462+35G>A) and intron 14 (c.1435–40G>C) are in strong linkage disequilibrium, as observed in both patients and controls.

Because genotype distributions of the c.972G>C (p.Q324H) variant were significantly different between cases and controls, we analyzed a second sample of 63 patients. Comparison of this new cohort of 113 patients with 116 controls revealed no deviation from HWE and no significant difference in genotype frequencies (69 GG, 41 GC, and 6 CC genotypes in controls and 79 GG, 31 GC, and 3 CC genotypes in cases; $P = 0.105$).

Except for c.157+30A>G, the genotype distribution of all other variants did not deviate from HWE. Analysis of c.157+30A>G in a second sample of 96 controls did not confirm this data, suggesting that the observed deviation from Hardy-Weinberg equilibrium was an effect of the recruited study sample (data not shown).

SIFT analysis with inclusion of the human *MYH* sequence and 27 sequences closely related from various

species defined the common polymorphisms p.V22M and p.Q324H as tolerated amino acid changes ($P = 0.07$ and 0.23, respectively). In contrast, the novel mutation p.Q484H was predicted as a not tolerated (ie, functionally significant) amino acid substitution with a SIFT probability of 0.02. Notably, both pathogenic mutations p.Y165C and p.G382D were predicted not to be tolerated with $P < 0.01$. In addition, the assumed polymorphism p.S501F was also predicted as not tolerated ($P = 0.02$).

Discussion

To date, it is impossible to reliably distinguish patients with HNPCC from "sporadic" cases through clinical and anamnestic data, because neither presents with indicative biomarkers such as diffuse polyposis or unusual stigmata.[1] Therefore, diagnosis is based on young age of disease onset and family history (Revised Bethesda Guidelines) and is confirmed by the detection of a germline mutation in one of the MMR genes *MSH2, MLH1, MSH6,* or *PMS2*.[1–4] Based on the knowledge that the vast majority of tumors from HNPCC patients present a MSI-H phenotype, which occurs in only 15% of sporadic tumors, molecular genetic testing is aimed at the identification of MSI in tumor tissue.[4] The recently Revised Bethesda Guidelines recommend which tumors should be tested for microsatellite instability.[5]

However, there is a diagnostic gap in those patients meeting the Bethesda guidelines but carrying microsatellite stable tumors. Aiming to find an inherited causative factor, we analyzed *MYH* as our candidate gene because clinical features of some patients with a biallelic *MYH* mutation are similar to certain patients suspected of HNPCC, ie, they present at young age of onset and/or with a family history and with few polyps.[9]

In most cases, the clinical findings of patients carrying biallelic pathogenic *MYH* mutations are similar to the attenuated familial adenomatous polyposis.[9] Sieber et al[9] found biallelic *MYH* mutations without an *APC* mutation in about one-third of the patients with multiple adenomas (15 to 100) and in only 5% of those patients with less than 15 polyps. Furthermore, biallelic *MYH* mutations were found in 7.5% of polyposis patients (ie, with more than one hundred polyps).

In contrast, Wang et al[15] recently reported two patients mistakenly thought to be HNPCC by presenting only with colorectal cancer. Further analysis revealed biallelic *MYH* mutations in both cases (one homozygous p.Y165C and one compound heterozygous p.Y165C/p.G382D). These findings are consistent with a recently published study from Croitoru et al[8] who analyzed a population-based series consisting of 1238 colorectal cancer patients for *MYH* mutation and found that the increased risk of colorectal cancer in mutation carriers was not consistently associated with the development of multiple adenomatous polyps. These data suggest that the number of adenomas itself does not favor or exclude *MYH* mutations in patients suspected of a hereditary condition. The age of presentation of patients with a *MYH* mutation ranged from 45 to 59 years with a median of 56 in the study of Sieber et al[9]; one-half of the mutation carriers had developed a colorectal cancer at the time of study, and more than 80% had a family history of colorectal cancer.

The age of onset in our study group ranged from 22 to 75 years with a median of 40 years, and patients were recruited through Revised Bethesda Guidelines. Because we have excluded all those with a vertical transmission of the disease, most patients had a carcinoma at young age, whereby the number of additional adenomas was low and less than five in all cases, excluding an attenuated polyposis.

In contrast to other studies, our analysis of *MYH* was not restricted to the most common mutations in Caucasian patients, namely p.Y165C and p.G382D. Instead, we performed a complete genomic sequence analysis of DNA from normal tissue so that we could test the suggestion of a study by Miyaki et al[16] that there might be ethnic and genetic differences in *MYH* mutations, leading to an underestimation of pathogenic *MYH* mutation.

A recently published population-based association study from Scotland including 2239 colorectal cancer cases and 1845 controls showed that biallelic *MYH* mutations account for 0.8% of cases aged <55 years. Notably, most biallelic carriers had coexisting adenomatous polyps, but 36% of biallelic carriers had no polyps.[17] There were no biallelic mutation carriers for the Y165C and G382D mutations in 1808 controls. Similar data have been obtained by a Canadian study that detected 24 biallelic *MYH* mutations in 1238 patients and no mutation in 1255 control subjects.[8]

In summary, we did not find biallelic pathogenic *MYH* mutations in young patients suspected of HNPCC without vertical transmission of disease. We found one patient with a heterozygous missense mutation (c.1452G>C, p.Q484H) in exon 15, which has not been previously described and was not detected in 116 healthy controls. In addition, another patient carried the p.G382D missense mutation in a heterozygous state. We conclude that the analysis of *MYH* cannot fill the diagnostic gap in those young patients suspected of HNPCC with microsatellite stable tumors and low numbers of adenomas.

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