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MUTYH and the mismatch repair system

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Published in:
HUMAN GENETICS

DOI:
[10.1007/s00439-005-0118-5](https://doi.org/10.1007/s00439-005-0118-5)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2006

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Niessen, RC., Sijmons, RH., Ou, J., Olthof, SGM., Osinga, J., Ligtenberg, MJ., Hogervorst, FBL., Weiss, MM., Tops, CMJ., Hes, FJ., de Bock, GH., Buys, CHCM., Kleibeuker, JH., & Hofstra, RMW. (2006). MUTYH and the mismatch repair system: partners in crime? *HUMAN GENETICS*, 119(1-2), 206-211. <https://doi.org/10.1007/s00439-005-0118-5>

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***MUTYH* and the mismatch repair system: partners in crime?**

Received: 15 September 2005 / Accepted: 3 December 2005 / Published online: 12 January 2006
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Abstract Biallelic germline mutations of *MUTYH*—a gene encoding a base excision repair protein—are associated with an increased susceptibility of colorectal cancer. Whether monoallelic *MUTYH* mutations also increase cancer risk is not yet clear, although there is some evidence suggesting a slight increase of risk. As the *MUTYH* protein interacts with the mismatch repair (MMR) system, we hypothesised that the combination of a monoallelic *MUTYH* mutation with an MMR gene mutation increases cancer risk. We therefore investigated the prevalence of monoallelic *MUTYH* mutations in carriers of a germline MMR mutation: 40 carriers of a

truncating mutation (group I) and 36 of a missense mutation (group II). These patients had been diagnosed with either colorectal or endometrial cancer. We compared their *MUTYH* mutation frequencies with those observed in a group of 134 Dutch colorectal and endometrial cancer patients without an MMR gene mutation (0.7%) and those reported for Caucasian controls (1.5%). In group I one monoallelic *MUTYH* mutation was found (2.5%). In group II five monoallelic germline *MUTYH* mutations were found (14%), four of them in *MSH6* missense mutation carriers (20%). Of all patients with an MMR gene mutation, only those with a missense mutation showed a significantly higher frequency of (monoallelic) *MUTYH* mutations than the Dutch cancer patients without MMR gene mutations ($P=0.002$) and the published controls ($P=0.001$). These results warrant further study to test the hypothesis of mutations in MMR genes (in particular *MSH6*) and *MUTYH* acting together to increase cancer risk.

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The *MUTYH* protein is a base excision repair (BER) DNA glycosylase that excises misincorporated adenines opposite 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxoG), which is a product of oxidative DNA damage (Slupska et al. 1999). Recently, it has been shown that having germline mutations in both copies (i.e. biallelic) of the *MUTYH* gene, increases the risk to develop multiple adenomatous polyps and, hence, colorectal cancer (Al Tassan et al. 2002). Whether monoallelic *MUTYH* mutations also increase cancer risk is not yet clear, although there is some evidence suggesting a slight increase of risk (Croitoru et al. 2004; Farrington et al. 2005). It is conceivable that germline monoallelic *MUTYH* mutations combined with germline mutations in other genes more strongly increase cancer risk.

Germline mutations of *MLH1*, *MSH2* and *MSH6* in combination with (functional) loss of the wild-type allele are known to cause a deficient DNA mismatch repair (MMR) system, and are responsible for hereditary

non-polyposis colorectal cancer (HNPCC). HNPCC is one of the most common inherited cancer syndromes and is characterised by susceptibility to develop colorectal cancer and extracolonic malignancies, in particular endometrial cancer (Lynch and de la Chapelle 2003).

Gu et al. (2002) demonstrated that the MUTYH protein interacts with the MMR protein MSH6 which is part of the MSH2/MSH6 heterodimer (hMutS α). They showed that hMutS α stimulates the DNA binding and glycosylase activities of MUTYH. In addition, it was shown that the interaction between MUTYH and MSH6 was weaker in cells that expressed wild-type *MSH2* and *MSH6* missense mutations (Gu et al. 2002). Considering this interaction between the MUTYH and MSH6 proteins, and assuming that part of (familial) cancer cases might be explained by combinations of low-penetrant germline mutations in known hereditary cancer genes, we hypothesised that a monoallelic *MUTYH* mutation might contribute to cancer susceptibility in carriers of an MMR gene (in particular *MSH6*) mutation. This might be most apparent in the case of missense MMR gene mutations, that in contrast to truncating mutations, “on their own” may result in a less severely impaired MMR system and may therefore less strongly contribute to cancer risk.

As a first step to test this hypothesis, we investigated the prevalence of monoallelic *MUTYH* mutations in patients diagnosed with colorectal and/or endometrial cancer who carried a truncating or missense MMR gene mutation in *MLH1*, *MSH2* or *MSH6*. We compared the frequencies of monoallelic *MUTYH* mutations in the two groups with that in a third group of patients, with colorectal and/or endometrial cancer without an MMR gene mutation and with the frequency of monoallelic *MUTYH* mutations reported for controls.

Materials and methods

Patient inclusion

In our study we included 210 unrelated colorectal and/or endometrial cancer patients (Table 1). Each patient had recently been tested for germline MMR gene mutations in one of four different genetic centres, i.e. the University Medical Center Groningen ($n=172$), the Radboud University Nijmegen Medical Center ($n=24$), the Netherlands Cancer Institute in Amsterdam ($n=5$) and the Leiden University Medical Center ($n=9$), because they had an increased risk for HNPCC, e.g. early-onset cancer, multiple HNPCC associated tumours, or a positive family history for these tumours. Seventy-six patients had a proven germline mutation in one of the MMR genes *MLH1*, *MSH2* and *MSH6* (36 missense and 40 truncating mutation carriers) and 134 patients had no MMR gene mutation. All patients had given their informed consent and the study was approved by the Medical Ethical Committee of the University Medical Center Groningen, The Netherlands.

Table 1 Patient characteristics

	CRC < 50 years	CRC > 50 years	END < 50 years	Multiple HNPCC associated tumours	Cases with positive first degree family history for HNPCC associated tumours	Cases from an Amsterdam II positive family	Cases with loss of MLH1, MSH2 or MSH6 staining in the tumour/total cases analysed ^a	Cases with one or more MSI-high tumour(s)/total cases analysed for MSI
Patients with truncating MMR gene mutation ($n=40$)	24/40 (60%)	12/40 (30%)	8/40 (20%)	15/40 (38%)	35/40 (88%)	27/40 (68%)	21/22 (95%)	26/29 (90%)
Patients with missense MMR gene mutation ($n=36$)	19/36 (53%)	14/36 (39%)	3/36 (8%)	3/36 (8%)	23/35 (66%)	8/35 (23%)	4/21 (19%)	10/31 (32%)
Patients without MMR gene mutation ($n=134$)	101/134 (75%)	34/134 (25%)	3/134 (2%)	42/134 (31%)	47/134 (35%)	7/134 (5%)	19/103 (18%)	55/133 (41%)
Total ($n=210$)	144/210 (69%)	60/210 (29%)	14/210 (7%)	60/210 (29%)	105/209 (50%)	42/209 (20%)	44/146 (30%)	91/193 (47%)

CRC colorectal cancer; END endometrial cancer; HNPCC hereditary non-polyposis colorectal cancer; MSI microsatellite instability
^a Those cases where immunohistochemical analysis was available and interpretable for MLH1, MSH2 and MSH6 staining

MUTYH mutation frequency in control population

Presently, no large groups of healthy Dutch controls have been screened for the entire *MUTYH* gene. Thus, the Dutch *MUTYH* mutation frequency is yet unknown. Therefore, data were taken from the only two studies in which the entire *MUTYH* gene of control individuals had been screened for mutations. Together, these studies reported 7 monoallelic *MUTYH* mutations in 461 controls from the British Isles [1.5% (95% CI 0.61–31)] (Sieber et al. 2003; Fleischmann et al. 2004).

Genetic analysis

For the mutation analysis in the MMR genes different techniques had been used: denaturing gradient gel electrophoresis (DGGE), single-strand conformational polymorphism analysis, direct sequencing and multiplex ligation dependent probe amplification, as described previously (Wu 1999; Gille et al. 2002).

Mutation analysis of *MUTYH* in DNA from peripheral blood lymphocytes was performed for 201 patients by DGGE combined with direct sequencing of PCR fragments showing aberrant patterns in DGGE. polymerase chain reaction (PCR) was performed in a total reaction volume of 50 µl containing 200 ng of

DNA, 15 pmol of each primer (Eurogentec, Serain, Belgium), 0.2 µl rTaq polymerase (5,000 U/ml, Amersham Pharmacia Biotech Inc., Biscataway, NY, USA), 5 µl DMSO (Sigma, Taufkirchen, Germany) and 5 µl PCR buffer (Amersham Biosciences, Roosendaal, Netherlands). The primer sequences are described in Table 2.

A stepdown cycling regime was performed in an Applied Biosystems GeneAmp 9700 Thermal Cycler (Foster City, USA) using the following conditions: initial denaturation for 3 min at 94°C, followed by five cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. Hereafter five more cycles with an annealing temperature of 57°C and 30 cycles with an annealing temperature of 54°C were performed. Finally, 5 min elongation at 72°C. PCR products were checked on a 2% agarose gel. PCR products with different melting temperatures were pooled in six different pools and PCR products were denaturated at 96°C for 10 min and re-annealed at 50°C for 45 min before gel electrophoresis to encourage the formation of heteroduplex molecules. For DGGE analysis of *MUTYH*, a 30 µl pooled sample was applied onto a 1-mm-thick 9% polyacrylamide (PAA) gel (acrylamide:bisacrylamide = 37.5:1) containing a denaturing gradient of 40–85% urea-formamide (100% urea-formamide contains 7 mol/l urea and 40% deionised formamide). Electrophoresis was performed in

Table 2 Primer sequences for mutation analysis in *MUTYH*

Exon		Primer 5' → 3'	Product size	Melting temperature (°C)
1	F	[60GC] CTTCCCTCTCCCAGAGC	293	81.7
	R	[CGCCGC] GACGTCTGAACGGAAGTTCG		
2	F	[40GC] CCCTTGGAAGGCCTCAAAAT	294	75.9
	R	[CGCGCGCG] CCAGCCTGAATCTGCCTTTC		
3	F	[CGC] CTGTGTCCCAAGACCCTGAT	299	76.5
	R	[40GC][ATATATAT] CACCCACTGTCCCTGCTC		
4	F	[40GC][ATATATAT] ACCATGGAGAAGACGGGTAG	299	77.4
	R	[CGCGC] GGTGGCATGAGGACACTG		
5,6	F	GTAGGGGCAGGTCAGCAGT	394	75.1
	R	[40GC] TCACCCGTCAGTCCCTCTAT		
7	F	[CGCCCG] CGGGTGATCTCTTTGACCTC	248	75.8
	R	[40GC] CCCCCTAGCTCCTCTACCAC		
8	F	[40GC] CCAGGAGTCTTGGGTGTCTT	280	77.8
	R	AGAGGGGCCAAAGAGTTAGC		
9	F	[CGCCC] CAGCCAGGCTAACTCTTTG	233	78.0
	R	[40GC] AGCAGAGCTCCTTTGCAGAC		
10	F	[CGCGCGCGCG] GTGTCTGCAAAGGAGCTCTG	308	78.5
	R	[50GC] CATAGGGCAGAGTCACTCCTTAG		
11	F	[40GC] GGGGCAGTGAGAAGTCTTAAG	239	77.1
	R	[CGCCCG] AAGAACTGGAATGGGGCTTC		
12	F	[CGCCCG] CCTAAAGCCCTCTTGGCTTG	346	77.9
	R	[40GC] CCGATTCCTCCATTCTCTC		
13	F	[CGCGCGC] AAGAGAGAATGGAGGGAATCG	292	79.4
	R	[60GC] TAGCCTCAAAAGCCAACATC		
14a	F	[40GC] CACCTTGGGGAGGTAAGTGA	202	74.0
	R	[CGCGCGCGCG] AGGCCAGCCCATATACTTGA		
14b	F	[CGCGCGCGCG] CCAGGTTGTCCACACCTTCT	255	75.9
	R	[40GC] GAACATGTAGGAAACACAAGGAA		
15	F	[CGCCC] AAAAAGTGCCAGCCCTCAC	237	73.8
	R	[40GC] AGTGAAGCCTGGAGTGGAGA		
16	F	[CGCCC]CCCTCCCCCAACTACAAGG	266	75.0
	R	[40GC] TTTACTAACAACAGGATTCTCAGG		

0.5×TAE (1×TAE = 40 mM Tris, HAC pH 8.0; 20 mM NaAc; 1 mM Na₂EDTA), at 120 V/19 cm, for 16 h at 62°C for exons 1 and 13 and at 60°C for the other exons using an INGENYphorU system (Ingeny International, Goes, The Netherlands). The gel pattern was visualised by ethidiumbromide staining for 10 min and UV transillumination of the gel. Exons displaying variant gel patterns were amplified again from genomic DNA with primers containing a universal M13 tail (forward primer: cgacgttgtaaacgacggccagt, reverse primer: caggaacagc-tatgac), cycle sequenced in both directions with dye labelled primers (Big Dye Terminator v3.1 Sequencing Kit, Applied Biosystems, Foster City, USA). In nine patients only exons 6–13 were screened by direct sequencing. Whenever, a sequence variant was detected, 200 control chromosomes of anonymous healthy Dutch individuals were screened for this variant.

Microsatellite instability analysis

MSI analysis was performed as described previously (Berends et al. 2002). Cancers were classified as MSI-high when two or more markers showed MSI and as MSI-low when one or no marker showed MSI. As a limited number of markers was analysed, the classification microsatellite-stable is not used.

Immunohistochemistry

Immunohistochemistry for the MLH1, MSH2 and MSH6 proteins was performed as previously described (Berends et al. 2002). Protein expression in normal tissue adjacent to the cancer served as internal positive control. The sections were scored as either negative (i.e., absence of detectable nuclear staining of cancer cells) or positive for MLH1, MSH2 and MSH6 staining. Scoring of the staining was performed without knowledge of the MSI or mutation status.

Statistical analysis

MUTYH mutation frequencies and two-sided 95% CI intervals were calculated for the different groups of MMR gene mutation carriers. To compare these frequencies with the frequency of monoallelic *MUTYH* mutations in controls reported in the literature, and with the frequency of monoallelic *MUTYH* mutations in colorectal and/or endometrial cancer patients without MMR gene mutation, crosstabs were constructed and results were analysed by the Fisher's Exact Test.

Results

One of the 134 cancer patients without an MMR gene mutation carried an *MUTYH* mutation [0.7% (95% CI.

0.019–4.1%)]. Amongst the 36 missense MMR gene mutation carriers, five monoallelic *MUTYH* mutations were detected [14% (4.7–29%)]. This frequency was significantly different from the *MUTYH* mutation frequency in the group of cancer patients without an MMR mutation ($P^{\text{non-mutation carriers}}=0.002$), and from the frequency (1.5%) in controls ($P^{\text{controls}}=0.001$). Interestingly, four of the *MUTYH* mutations were found amongst the 20 patients with an *MSH6* missense mutation [20% (5.7–44); $P^{\text{non-mutation carriers}}=0.001$; $P^{\text{controls}}=0.001$] and one mutation was found amongst the five *MSH2* missense mutation carriers [20% (0.51–72); $P^{\text{non-mutation carriers}}=0.071$; $P^{\text{controls}}=0.083$]. No *MUTYH* mutations were found in DNA from any of 11 *MLH1* missense mutation carriers [0% (0.0–35)]. In the group of 40 truncating MMR gene mutation carriers a single monoallelic *MUTYH* mutation was found [2.5% (0.1–13); $P^{\text{non-mutation carriers}}=0.408$; $P^{\text{controls}}=0.489$]. The *MUTYH* mutation frequency in the group of cancer patients without an MMR mutation, was not significantly different from the 1.5% reported in British controls ($P=0.430$). None of the *MUTYH* mutations was detected in 200 control chromosomes. The results of the mutation analysis are summarised in Table 3. In addition to the *MUTYH* mutations, several previously in the normal population described polymorphisms were detected. In the group of patients where *MUTYH* ($n=201$) was analysed by DGGE, the p.Val22Met (c.64G>A), p.Gln324His (c.972G>C) and p.Ser501Phe (c.1502C>T) polymorphisms were found with frequencies of 12.4%, 29.9%, and 3.0%, respectively. In addition, a silent mutation p.Tyr90Tyr (c.270C>T) was detected and various changes in non-coding sequences: c.36+75C>G (0.5%), c.157+30A>G (6.5%), c.346+56G>A (1.0%), c.462+35G>A (10.4%), c.648+21C>A (1.0%), c.1145–27C>T (1.0%).

Discussion

In the present study we found a significantly higher frequency of monoallelic *MUTYH* mutations in (*MSH6*) missense mutation carriers than in published Caucasian controls and Dutch colorectal and endometrial cancer patients without MMR gene mutation.

We observed five different *MUTYH* mutations: four missense mutations, one splice site mutation. Of these, the p.Gly382Asp and p.Tyr165Cys missense mutations are the two most common pathogenic *MUTYH* missense mutations found in patients with multiple adenomatous polyps. The sequence change caused by the mutation c.36+1G>A interferes with the splice donor site and is considered pathogenic. For the two other missense mutations, p.Arg295Cys and p.Asp91Asn, no hard evidence for their pathogenicity is yet available. What we do know is that none of the mutations in this study was present in the 200 control chromosomes we examined. Furthermore, Sieber et al. (2003) reported the p.Arg295Cys mutation in a patient with three colorectal

Table 3 Combinations of an *MUTYH* mutation and an MMR gene mutation occurring in individual patients

Patient	Gene	Exon	Nucleotide change	Codon	Change	Cancers/age of diagnosis	FH/ACII	MSI	IHC analysis MLH1/MSH2/MSH6
Group I									
1.	MUTYH MSH2	7 1-3	A → G at 493 Deletion of exons 1-3	165	Tyr → Cys	BL/56 CRC/58 CRC/58 END/59	-/-	H H L	+/-/- +/-/-
Group II									
2.	MUTYH MSH2	IVS1 11	G → A at 36 + 1 T → C at 1729		Splice defect Ile → Thr	CRC/52 CRC/65	-/-	L	-/+ / +
3.	MUTYH MSH6	7 4	A → G at 494 C → G at 1186	165 396	Tyr → Cys Leu → Val	CRC/47	+/-	L	+ / + / +
4.	MUTYH MSH6	10 2	C → T at 883 G → T at 431	295 144	Arg → Cys Ser → Ile	CRC/48	-/-	L	+ / + / +
5.	MUTYH MSH6	10 5	C → T at 883 C → T at 3259	295 1,087	Arg → Cys Pro → Ser	CRC/39	+/-	L	+ / + / +
6.	MUTYH MSH6	13 2	C → A at 1145 G → T at 431	382 144	Gly → Asp Ser → Ile	END/45	+/-	H	+ / + / +
Group III									
7.	MUTYH	3	G → A at 271	91	Asp → Asn	CRC/64 CRC/69	-/-	L	NA/NA/NA

Group I: 40 carriers of a truncating MMR gene mutation (14 *MLH1*, 21 *MSH2*, 5 *MSH6*); Group II: 36 carriers of a missense MMR gene mutation (11 *MLH1*, 5 *MSH2*, 20 *MSH6*); Group III: 134 cancer patients without an MMR gene mutation
CRC colorectal cancer; *END* endometrial cancer; *BL* bladder cancer; *FH* positive first degree family history for HNPCC associated tumours; *ACII* Amsterdam II positive; *MSI* microsatellite instability; *H* high; *L* low; *IHC* immunohistochemistry; *NI* not interpretable; *NA* not analysed

adenomas, and the p.Asp91Asn mutation has been reported once in a patient with colorectal cancer and 30 adenomas (Nielsen et al. 2005), indicating that these two missense mutations might be pathogenic.

Of course one might also question the pathogenic nature of the four MMR missense variants that are found in combination with an *MUTYH* mutation. The *MSH2* p.Ile577Thr and the *MSH6* p.Pro1087Ser mutations have not been reported before, and we detected the latter in an other patient with colorectal cancer at age 36 (data not published). Alternative amino acid substitutions at this residue have been described (Wijnen et al. 1999) (<http://www.insight-group.org>). The pathogenicity of missense mutations is difficult to establish, even with help of functional assays. For example, Kolodner et al. performed a *S. cerevisiae*-based assay to assess the pathogenicity of the *MSH6* p.Ser144Ile missense mutation, and suggested that this mutation might be pathogenic (Kolodner et al. 1999). In contrast, in an in-vitro MMR assay that studied the ability of the mutant proteins to form MSH2-MSH6 heterodimers, the same *MSH6* mutation appeared as functional as the wild type (Kariola et al. 2002). Some mutations we found may eventually turn out to be harmless polymorphisms. For instance, the *MSH6* p.Leu396Val mutation has been reported not only in cancer patients, but also in healthy controls (Wijnen et al. 1999) and was not considered pathogenic in a functional assay (Kolodner et al. 1999). However, it is conceivable that a missense mutation on its own has little or no effect, but that it can significantly contribute to cancer risk in the presence of germline mutations in other genes.

We compared the *MUTYH* mutation frequency in the two groups of mutation carriers with the *MUTYH* mutation frequency in cancer patients without MMR mutation and with the 1.5% mutation frequency reported for controls. The *MUTYH* mutation frequency in the group of cancer patients without MMR mutation turned out to be not significantly different from the frequency in healthy British controls. In the former group, we expected the *MUTYH* mutation frequency to be similar or (somewhat) higher than in the normal British and Dutch population. Given the fact that there is presently no evidence to suggest a protective effect of *MUTYH* mutations against cancer, we have no reason to assume that the frequency in the normal Dutch population will be significantly higher than in Dutch cancer patients.

As co-occurrence of MMR gene missense mutations and *MUTYH* mutations might be a matter of chance, and considering the idea that probably most *MSH6* (missense) mutations alone are not strongly promoting CRC development, one would expect the co-occurrence of both mainly in sporadic cancers. In fact, none of the five patients with a *MUTYH* and an MMR gene missense mutation were from an Amsterdam II positive family and two of the five patients had a negative first degree family history for HNPCC-associated tumours. One of the five patients with a *MUTYH* and an MMR gene missense mutation had an MSI-high tumour. Although this might suggest that MMR deficiency did not underlie tumour development in four of these five patients, three of these four patients carried a *MSH6* mutation and pathogenic *MSH6* germline mutations are known to be associated with lower rates of microsatellite instability than germline

mutations in other MMR genes (Kolodner et al. 1999; Berends et al. 2002). From a theoretical point of view, the *MSH6* and *MUTYH* mutations might also have jointly impaired base-excision repair or impaired apoptosis signalling, a process that has been linked to MMR gene function (Fishel 2001).

Our findings seem to be contradictory to the study recently published by Ashton et al. (2005). They performed a similar study, in which they compared the *MUTYH* mutation frequencies in 233 MMR mutation negative and in 209 mutation positive HNPCC patients. They detected five heterozygous *MUTYH* mutations in the former and two in the latter group. There was no statistical difference in *MUTYH* mutation frequency between the two groups. However, we cannot compare their results with ours in all details, as for example, no *MSH6* mutation carriers were included in their study, no subgroup analysis of truncating and missense MMR gene mutation carriers was presented, and instead of full *MUTYH* gene analysis, only the frequencies of the two most common mutations were determined.

In conclusion, we present the statistical observation that *MUTYH* mutations occur more frequently in MMR gene missense mutation carriers than in controls. Our preliminary results suggest that the presence of a monoallelic *MUTYH* mutation combined with a missense MMR gene mutation, of *MSH6* in particular, leads to an increased cancer risk. As the possible interaction of the observed missense mutations is presently unknown at the functional level, we cannot reach any definite conclusions yet as to its clinical significance. However, if larger studies confirm these results, then this might have implications for management of patients with monoallelic *MUTYH* mutations. They should all be examined for mutations in *MSH6* and conversely, *MSH6* missense mutation carriers should all be screened for *MUTYH* mutations.

Acknowledgements The authors wish to thank Maran Olderode-Berends for the patient inclusion and Ying Wu, Neeltje Arts and Martijn Schliekelmann for the mutation detection on the mismatch repair genes. This work was supported by grants no. RUG 1997–1544 and RUG 2002–2678 of the Dutch Cancer Society.

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