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Puijenbroek, M. van

CHAPTER 4

Identification of (atypical) MAP patients by *KRAS2* c.34 G>T prescreening followed by *MUTYH* hotspot analysis in formalin-fixed paraffin-embedded tissue

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Identification of Patients with (Atypical) *MUTYH*-Associated Polyposis by *KRAS2* c.34G > T Prescreening Followed by *MUTYH* Hotspot Analysis in Formalin-Fixed Paraffin-Embedded Tissue

Marjo van Puijenbroek,[1] Maartje Nielsen,[2] Carli M.J. Tops,[2] Hans Halfwerk,[1] Hans F.A. Vasen,[3] Marjan M. Weiss,[2] Tom van Wezel,[1] Frederik J. Hes,[2] and Hans Morreau[1]

Abstract

Purpose: To assess the feasibility of identifying patients with (atypical) *MUTYH*-associated polyposis (MAP) by *KRAS2* c.34G > T prescreening followed by *MUTYH* hotspot mutation analysis in formalin-fixed paraffin-embedded tissue (FFPE).

Methods: We collected 210 colorectal FFPE tumors from 192 individuals who presented with <10 adenomas or familial mismatch repair proficient colorectal carcinomas with <10 concomitant adenomas. The tissues were tested for somatic *KRAS2* mutations and for three Dutch hotspot *MUTYH* germ line mutations (p.Tyr165Cys, p.Gly382Asp, and p.Pro391Leu) by sequencing analysis.

Results: The c.34G > T, *KRAS2* transversion was detected in 10 of 210 tumors. In one of these 10 cases, a monoallelic p.Gly382Asp *MUTYH* mutation was found and a full *MUTYH* analysis in leukocyte DNA revealed an unclassified variant p.Met269Val. This was in a 61-year-old patient with a cecum carcinoma and three adenomas. After further requests, her family case history revealed that her brother had had between 10 and 15 adenomas and turned out to carry both *MUTYH* germ line mutations. *MUTYH* hotspot mutation screening in 182 patients without the somatic c.34G > T *KRAS2* mutation led to the detection of three monoallelic germ line *MUTYH* mutation carriers.

Conclusion: *KRAS2* c.34G > T somatic prescreening, followed by *MUTYH* hotspot mutation analysis when positive, can identify patients with (atypical) MAP. If heterozygous hotspot *MUTYH* mutations are identified, a complete germ line *MUTYH* mutation screening should be carried out if possible. Immediate *MUTYH* hotspot mutation analysis is a practical alternative in patients with >10 adenomas or in cases of multiple colorectal carcinomas in one generation for which only FFPE tissue is available.

The aim of this study was to explore the feasibility of identifying patients with (atypical) MAP using *KRAS2* c.34G > T somatic prescreening followed by *MUTYH* hotspot analysis in patients that presented with <10 adenomas or familial mismatch repair proficient colorectal carcinomas (CRC) with <10 concomitant adenomas.

In 2002, the first autosomal recessive colorectal cancer and polyposis syndrome, *MUTYH*-associated polyposis (MAP), was described (1). Biallelic germ line *MUTYH* mutations predispose carriers to somatic G > T transversions in genes involved in the tumorigenesis of CRCs, such as *APC* and *KRAS2*, due to failure of base excision repair to remove the purine adenine aberrantly coupled to 8-oxo-guanine by DNA polymerase (1–4).

In most cases, patients with MAP develop between 10 and 500 polyps at a mean age of ~50 years (5–7). Previously, in large cohorts of patients with CRC (with or without polyps), ~1% of patients with biallelic MAP were detected, some of whom were without polyps (8, 9). Although in other cohorts of patients with <10 polyps, no *MUTYH* mutation carriers were detected (10), the question remains of how prevalent the (biallelic) *MUTYH* mutations are in familial CRC cases with <10 polyps, with or without concomitant CRC.

In the Netherlands, clinical geneticists advise diagnostic testing for *MUTYH* germ line mutations based on the number of adenomas, age at diagnosis, and the family history. *MUTYH* will be analyzed in patients with 10 to 100 adenomas at ages under 70 years, whereas in CRC patients with a history of <10 adenomas, Lynch syndrome could also be considered. In patients with classic polyposis (>100 adenomas), germ line *APC* mutations can be excluded prior to *MUTYH* testing (11).

Authors' Affiliations: [1]Department of Pathology and [2]Center for Human and Clinical Genetics, Leiden University Medical Center, and [3]The Netherlands Foundation for the Detection of Hereditary Tumors, Leiden, the Netherlands

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Requests for reprints: Hans Morreau, Department of Pathology, Leiden University Medical Center, Building L1Q, P.O. Box 9600, 2300 RC Leiden, the Netherlands. Phone: 31-71-526-6630; Fax: 31-71-524-8158; E-mail: J.Morreau@lumc.nl.

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Table 1. Basic clinical characteristics of the familial microsatellite stable cases

	No. of patients	Carcinomas			Adenomas		
		Right	Left	Unspecified	<5	5-10	>10
Adenoma <40 y	7	—	—	—	6	1	—
Adenoma 40-50 y	14	—	—	—	13	1	—
Adenoma >50 y	18	—	—	—	17	1	—
Carcinoma <50 y	74	18	46	10	8	1	—
Carcinoma >50 y	79	18	48	13	18	1	1*

*Patient, at 71 years old; left-sided colon carcinoma, no polyps identified and at 77 years old; right-sided colon carcinoma and 10 to 20 polyps (therefore not immediately eligible for germ line *MUTYH* testing).

Two missense mutations (p.Tyr165Cys and p.Gly328Asp) account for 73% of the *MUTYH* mutations that have been reported thus far (12). In addition, there seems to be population-specific *MUTYH* mutations, such as the Italian 1395delGGA, the Portuguese 1186-1187insGG, and the Indian p.Glu466OCHer (5, 10, 13). In the Netherlands, we identified p.Pro391Leu as a possible founder mutation. Three hotspot mutations (p.Tyr165Cys, p.Gly328Asp, and p.Pro391Leu) represent 89% of the *MUTYH* mutations that are found in Dutch patients with MAP, and at least one of these mutations is present in all biallelic germ line *MUTYH* mutation carriers of Dutch origin identified thus far, and 79% of these carriers have two hotspot mutations (7). Up to 64% of MAP carcinomas showed a specific G > T transversion in *KRAS2* c.34G > T, p.Gly12Cys (3, 4). The latter somatic mutation is infrequent in consecutive series of sporadic CRC (14).

Materials and Methods

Patient cohort. We analyzed 210 tumors from 192 patients who were referred to the Department of Pathology, as part of the familial cancer clinics, and who presented with <10 adenomas or familial mismatch repair proficient CRCs with <10 concomitant adenomas. Microsatellite instability analysis and additional immunohistochemistry was done in order to exclude a mismatch repair gene defect.

Basic clinical characteristics of these familial cases are summarized in Table 1. Complete pedigree information was available in only 62 cases (data not shown). Informed consent was obtained for DNA testing according to protocols approved by the local ethics review boards, and the cases were analyzed following the medical ethnical guidelines described in the Code for Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Sciences.[4]

DNA isolation. Genomic DNA of normal colon and colorectal tumor tissue was extracted from formalin-fixed paraffin-embedded (FFPE) material as described by De Jong et al. (15). Microsatellite analysis was done as described (15).

Somatic KRAS2 mutation analysis. Nested *KRAS2* mutation analysis (16), and an improved *KRAS2* mutation analysis was used (preventing the amplification of chromosome 6 *KRAS2* pseudogene sequences; detailed information will be given on request).

Somatic APC mutation analysis. Samples were screened for the presence of mutations in the mutation cluster region codons 1286-1513 of *APC* by sequence analysis as previously described (16).

Dutch MUTYH mutation hotspot (p.Tyr165Cys, p.Gly382Asp, and p.Pro391Leu) analysis in FFPE material. Mutation analysis was done by direct sequencing of a PCR product which was obtained under standard PCR conditions. The following primer sets were developed: forward 5′-CCC ACA GGA GGT GAA TCA ACT-3′, and reverse 5′-GTT CCT ACC CTC CTG CCA TC-3′ for *MUTYH* (p.Tyr165Cys), and forward 5′-GGC AGT GGC ATG AGT AAC AAG-3′ and reverse 5′-CTT GCG CTG AAG CTG CTC T-3′ for *MUTYH* (p.Gly328Asp) and (p.Pro391Leu).

Germ line MUTYH mutation analysis. When a *KRAS2* c.34G > T mutation was found, or when *MUTYH* hotspot analysis showed a monoallelic *MUTYH* mutation, mutation analysis of the whole *MUTYH* gene was done in leukocyte DNA (when available) as described by Nielsen et al. (7). For further details, see the LUMC web site.[5]

Results

Frequency of somatic KRAS2 mutations. We identified 34% (54 of 159) and 27% (14 of 51) *KRAS2* mutations in mismatch repair proficient carcinomas and adenomas, respectively (Table 2). The majority of carcinomas showed G > A transitions (36 of 54, 67%), of which 75% (27 of 36) were c.35G > A transitions. G > T transversions were detected in 26% (14 of 54), whereas G > C transitions were detected in only 6% (3 of 54) of the carcinomas. Preferential occurrence of G > A transitions over G > T transversions was not seen in adenomas (6 of 10 versus 7 of 10, respectively), although we only had a low number of cases.

Cases with somatic KRAS2 c.34G > T transversions. The c.34G > T, p.Gly12Cys *KRAS2* mutation was detected in 10 cases (six carcinomas, four adenomas; Table 3). Six of the 10 showed inactivating *APC* somatic mutations other than G > T transversions (Table 3). One patient with a somatic c.34G > T *KRAS2* mutation in her carcinoma carried a monoallelic p.Gly382Asp germ line *MUTYH* mutation, and subsequent complete germ line *MUTYH* analysis in leukocyte-derived DNA revealed an unclassified variant c.805A > G, p.Met269Val. No somatic *APC* mutation was found. This female patient (III.1) presented with a right-sided cecum carcinoma and three adenomas at 61 years old. Her pedigree is shown in Fig. 1. Only after further requests did her family case history reveal that her brother (living abroad) had had between 10 and 15 adenomas and turned out to carry both *MUTYH* germ line mutations (III.2). The nine remaining cases with c.34G > T *KRAS2* mutations showed no hotspot *MUTYH* mutations in FFPE material. Leukocyte DNA was available in three of nine

[4] http://www.federa.org/?s=1&m=78&p=&v=4

[5] http://www.lumc.nl/4080/DNA/MUTYH.html

Table 2. Somatic mutation analysis of codons 12 and 13 of *KRAS2*

	Patients	Carcinomas (159)		Adenomas (51)	
		% *KRAS2* mutations	No. of *KRAS2* mutations	% *KRAS2* mutations	No. of *KRAS2* mutations
Familial MRR proficient	192	(54) 34%	1 (c.34G > A) + (=) 6 (c.34G > T) + (=) 2 (c.34G > C) + (=) 27 (c.35G > A) + (=) 1 (c.35G > C) + (=) 8 (c.35G > T) + (=) 9 (c.38G > A) + (=)	(14) 27%	4 (c.34G > T) + (=) 4 (c.35G > A) + (=) 1 (c.35G > C) + (=) 3 (c.35G > T) + (=) 2 (c.38G > A) + (=)

Abbreviations: ca, carcinoma; ad, adenoma; (=), wild-type.

cases to complete *MUTYH* germ line mutation analysis but showed no *MUTYH* mutations.

MUTYH *germ line hotspot mutation carriers without a somatic* KRAS2 *c.34G > T transversion.* In 182 patients without the c.34G > T *KRAS2* mutation, *MUTYH* hotspot analysis revealed three heterozygotes: two with the p.Gly382Asp mutation and one with the p.Tyr165Cys mutation. The complete *MUTYH* gene could be analyzed in two of the three patients, but no additional mutation was detected. One of the two heterozygous p.Gly382Asp patients (not fully tested for *MUTYH*) carried a somatic c.35G > A mutation in *KRAS2* in his tumor. He presented with a well-differentiated right-sided adenocarcinoma when he was 74 years old. The second patient (fully tested for *MUTYH*) with the monoallelic *MUTYH* p.Gly382Asp mutation had no mutation in *KRAS2* in his tumor and presented with a rectal carcinoma at age 41 years. The third patient (fully tested for *MUTYH*), with a monoallelic p.Tyr165Cys *MUTYH* mutation, presented with five adenomas at age 43 years, three of which were tested and showed no somatic *KRAS2* mutations.

Discussion

Because MAP carcinomas show a specific c.34G > T *KRAS2* mutation (2–4), we investigated whether somatic *KRAS2* prescreening could be used to detect patients with atypical MAP among individuals who presented with <10 adenomas or with familial mismatch repair proficient CRCs with <10 or no concomitant adenomas. For the same purpose, we did *MUTYH* hotspot analysis in FFPE material. In the Netherlands, it is logical to search for hotspot *MUTYH* mutations because MAP patients of Dutch origin always have at least one of the hotspot mutations (data not shown). If a *MUTYH* hotspot mutation

Table 3. Patients with c.34G > T, p.Gly12Cys mutations

Patient ID	Age of onset (y)	Tumor	MSI	Germ line *MUTYH* mutation	Somatic *KRAS2* mutation	Somatic *APC* mutation	
						Nucleotide change	Amino acid change
1	35	Sigmoid carcinoma	S	wt*	(c.34G > T) + (=)†	(c.4468delC) + (=)‡	(p.His1490fs) + (=)
2 T1	35	Cecum adenoma	S	wt	(c.34G > T) + (=)†	(c.4497delA) + (=)‡	(pSer1501fs) + (=)
2 T2	35	Cecum carcinoma	S	wt	wt	wt‡	
3	49	Cecum adenoma	S	wt*	(c.34G > T) + (=)†	(c.4285C > T) +(=)§	(p.Gln1429X) + (=)
4	40	Sigmoid adenoma	S	wt	(c.34G > T) + (=)†	(c.4285C > T) + (=)	(p.Gln1429X) + (=)
5	71	Sigmoid carcinoma	S	wt*	(c.34G > T) + (=)†	wt‡	
6	47	Cecum adenoma	S	wt*	(c.34G > T) + (=)†	wt§	
7	45	Sigmoid carcinoma	S	wt	(c.34G > T) + (=)†	(c.3922_3929del AAAGAAAA) + (=)	(p.Lys1308fs) + (=)
8	45	Sigmoid carcinoma	S	wt*	(c.34G > T) + (=)†	wt§	
9	51	Cecum carcinoma	S	wt*	(c.34G > T) + (=)†	(c.3949G > C) + (=)§	(p.Glu1317Gln) + (=)
10	61	Cecum carcinoma‖	S	(c.805A > G) + (c.1145G > A)¶	(c.34G > T) + (=)†	wt§	

Abbreviations: MSI, microsatellite instability; S, stable; wt, wild-type; T1, tumor 1; T2, tumor 2; (=), wild-type.
*Patients were only tested for three *MUTYH* hotspots (p.Tyr165Cys, p.Gly382Asp, and p.Pro391Leu).
†(c.34 G > T, p.Gly12Cys) + (=).
‡SNP rs 41115 (c.4479G>A) + (=) confirmed in normal DNA.
§SNP rs 41115 (c.4479G>A) + (c.4479G > A) confirmed in normal DNA.
‖This patient also presented with three adenomas.
¶(c.805A > G,p.Met269Val) + (c.1145G > A, p.Gly382Asp).

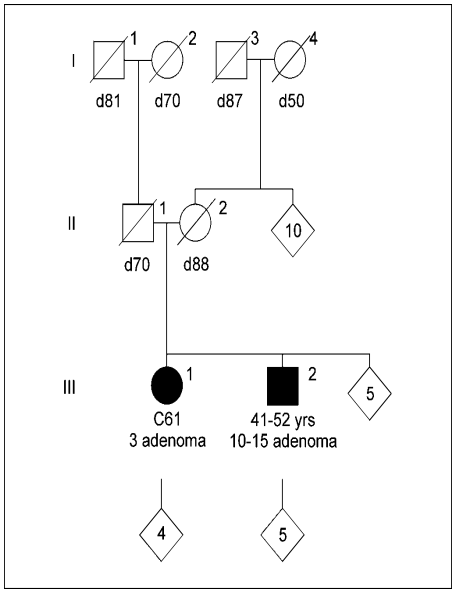

Fig. 1. Pedigree of a Dutch family in which two members were found to carry a heterozygous pGly382Asp germ line *MUTYH* mutation and an unclassified variant of *MUTYH*, c805A > G, pMet269Val. C, colorectal cancer; d, age at death.

is present, the gene should be screened for additional rare mutations in *MUTYH*.

This study identified one compound heterozygote *MUTYH* mutation carrier (p.Gly382Asp, p.Met269Val) with *KRAS2* mutation screening for the specific c.34G > T somatic mutation and three other monoallelic *MUTYH* germ line mutation carriers with the *MUTYH* hotspot analysis.

In our total cohort of 192 cases, 10 tumors had a somatic c.34G > T *KRAS2* mutation (six carcinomas and four adenomas). Of these, one turned out to carry a germ line *MUTYH* mutation, although this patient would a priori not have been tested for *MUTYH* mutations. This patient (and later her brother, who turned out to have >10 adenomas) carried both a proven pathogenic *MUTYH* mutation p.Tyr165Cys and an unclassified variant, c.805A > G, p.Met269Val. The c.805A > G, p.Met269Val unclassified variant in *MUTYH* was identified only after a full *MUTYH* gene mutation screening as a next step. This *MUTYH* unclassified variant described by Lejeune et al. is evolutionarily strongly conserved and locates within the adenine recognition motif (17). Although it was not predicted to be damaging by Polyphen software, the above family characteristics might suggest otherwise.

In the remaining nine patients with c.34G > T *KRAS2* somatic mutations, six also had inactivating *APC* somatic mutations. However, none of these mutations were G > T transversions and no germ line hotspot *MUTYH* mutations were identified.

In conclusion, we have shown that *KRAS2* c.34G > T, p.Gly12Cys somatic prescreening followed by *MUTYH* (hotspot) mutation analysis of cases (presenting with <10 adenomas or familial mismatch repair proficient CRCs with <10 or no concomitant adenomas) could be used successfully to identify patients with (atypical) MAP. If monoallelic (hotspot) *MUTYH* mutations are identified subsequently, full germ line *MUTYH* mutation analysis should also be carried out to exclude additional rare mutations. *KRAS2* c.34G > T prescreening only followed by *MUTYH* hotspot analysis when positive, is cost-effective especially when transformed into an allele-specific PCR. We estimate that the cost would be at least five times higher if immediate *MUTYH* hotspot mutation analysis would be done in all cases. The latter, however, is a practical alternative in patients with >10 adenomas or in family cases of multiple CRCs in one generation, for which only FFPE tissue is available.

Since finishing our study, we implemented *KRAS2* c.34G > T prescreening in our diagnostic setting. We recently identified a second atypical MAP family. The female index patient was diagnosed with metastasized colon cancer at age 41. No polyps were described. After identification of the c.34G > T transversion in *KRAS2* in her tumor, subsequent *MUTYH* hotspot analysis identified a monoallelic p.Gly382Asp *MUTYH* mutation. Full germ line *MUTYH* mutation analysis showed a 956-13 G > T splice variant.

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