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

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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 germline 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 germline 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 abnormally 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).
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.  

**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 PCR 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 mismatch repair cluster regions 1266-1513 of APC by sequence analysis as previously described (13).  

**Dutch MUTYH mutation hotspot (p.Tyr165Cys, p.Gly328Asp, 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’-CTT CAC CTC CAG CCA TC-3’ for MUTYH (p.Tyr165Cys), and forward 5’-GCC AGA AGT AGT AAC AAG-3’ and reverse 5’-CTT GCG 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.  

### 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.Gly328Asp 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 at 61 years old. Her pedigree is shown in Fig. 1.

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

<table>
<thead>
<tr>
<th>No. of patients</th>
<th>Carcinomas</th>
<th>Adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>Adenoma &lt;40 y</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td>Adenoma 40-50 y</td>
<td>14</td>
<td>—</td>
</tr>
<tr>
<td>Adenoma &gt;50 y</td>
<td>18</td>
<td>—</td>
</tr>
<tr>
<td>Carcinoma &lt;50 y</td>
<td>74</td>
<td>18</td>
</tr>
<tr>
<td>Carcinoma &gt;50 y</td>
<td>79</td>
<td>18</td>
</tr>
</tbody>
</table>

*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).

**Table 2. Frequency of somatic KRAS2 mutations.

<table>
<thead>
<tr>
<th>Carcinomas</th>
<th>Adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;5</td>
</tr>
<tr>
<td>G &gt; A</td>
<td>36</td>
</tr>
<tr>
<td>G &gt; T</td>
<td>14</td>
</tr>
<tr>
<td>G &gt; C</td>
<td>3</td>
</tr>
</tbody>
</table>

**Table 3. Cases with somatic KRAS2 c.34G > T transversions.

<table>
<thead>
<tr>
<th>Carcinomas</th>
<th>Adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;5</td>
</tr>
<tr>
<td>p.Gly12Cys</td>
<td>6</td>
</tr>
</tbody>
</table>

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

5 http://www.lumc.nl/4080/DNA/MUTYH.html
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 pre-screening 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>
<thead>
<tr>
<th>Patient ID</th>
<th>Age of onset (y)</th>
<th>Tumor</th>
<th>MSI</th>
<th>Germ line MUTYH mutation</th>
<th>Somatic KRAS2 mutation</th>
<th>Somatic APC mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>Sigmoid carcinoma</td>
<td>S</td>
<td>wt*</td>
<td>(c.34G &gt; T) + (=)</td>
<td>(c.4468deIC) + (=)</td>
</tr>
<tr>
<td>2 T1</td>
<td>35</td>
<td>Cecum adenoma</td>
<td>S</td>
<td>wt</td>
<td>(c.34G &gt; T) + (=)</td>
<td>(c.4497delA) + (=)</td>
</tr>
<tr>
<td>2 T2</td>
<td>35</td>
<td>Cecum carcinoma</td>
<td>S</td>
<td>wt</td>
<td>(c.34G &gt; T) + (=)</td>
<td>(c.4285C &gt; T) + (=)</td>
</tr>
<tr>
<td>3</td>
<td>49</td>
<td>Cecum adenoma</td>
<td>S</td>
<td>wt</td>
<td>(c.34G &gt; T) + (=)</td>
<td>(c.4285C &gt; T) + (=)</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>Sigmoid adenoma</td>
<td>S</td>
<td>wt</td>
<td>(c.34G &gt; T) + (=)</td>
<td>(c.4285C &gt; T) + (=)</td>
</tr>
<tr>
<td>5</td>
<td>71</td>
<td>Sigmoid carcinoma</td>
<td>S</td>
<td>wt*</td>
<td>(c.34G &gt; T) + (=)</td>
<td>(c.3922_3929delAAAGAAA) + (=)</td>
</tr>
<tr>
<td>6</td>
<td>47</td>
<td>Cecum adenoma</td>
<td>S</td>
<td>wt*</td>
<td>(c.34G &gt; T) + (=)</td>
<td>(c.3922_3929delAAAGAAA) + (=)</td>
</tr>
<tr>
<td>7</td>
<td>45</td>
<td>Sigmoid adenoma</td>
<td>S</td>
<td>wt</td>
<td>(c.34G &gt; T) + (=)</td>
<td>(c.3922_3929delAAAGAAA) + (=)</td>
</tr>
<tr>
<td>8</td>
<td>45</td>
<td>Sigmoid carcinoma</td>
<td>S</td>
<td>wt*</td>
<td>(c.34G &gt; T) + (=)</td>
<td>(c.3922_3929delAAAGAAA) + (=)</td>
</tr>
<tr>
<td>9</td>
<td>51</td>
<td>Cecum carcinoma</td>
<td>S</td>
<td>wt*</td>
<td>(c.34G &gt; T) + (=)</td>
<td>(c.3922_3929delAAAGAAA) + (=)</td>
</tr>
</tbody>
</table>

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).

1 (c.34 G > T, p.Gly12Cys) + (=).

1 SNP rs 41115 (c.4479G > A) + (=) confirmed in normal DNA.

1 SNP rs 41115 (c.4479G > A) + (c.4479G > A) confirmed in normal DNA.

1 This patient also presented with three adenomas.

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.

References

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