# Molecular Analysis of Colorectal Cancer Tumors from Patients with Mismatch Repair – Proficient Hereditary Nonpolyposis Colorectal Cancer Suggests Novel Carcinogenic Pathways

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**Abstract**

**Purpose:** A subset of colorectal cancers (CRC) arises in families that, despite fulfilling clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC), do not show evidence of a mismatch repair (MMR) deficiency. The main objective of this study was to characterize these tumors at the molecular level.

**Experimental Design:** After comprehensive germ line mutation scanning, microsatellite analysis, and MMR protein expressions, we selected a well-defined cohort of 57 colorectal tumors with no evidence of MMR defects. In this group of tumors, we analyzed *KRAS, BRAF,* and *APC* somatic mutations, as well as methylguanine methyltransferase (MGMT) and β-catenin expression. We correlated these alterations with clinicopathologic data and explored the relationship between *KRAS* G > A transitions and lack of MGMT expression.

**Results:** The mutation profile at the *RAS*/*RAF*/*MAPK* pathway mimics sporadic microsatellite-stable CRCs. We found an average age of diagnosis 10 years older in *KRAS*-mutated patients ($P = 0.001$). In addition, we show that *KRAS* G > A transitions are actively selected by tumors, regardless of MGMT status. Similarities with HNPCC high – microsatellite instability tumors are observed when *APC* data are analyzed. The *APC* mutation rate was low and small insertions/deletions accounted for 70% of the alterations. In addition, we found a low frequency of β-catenin nuclear staining. Finally, we did not find evidence of tumors arising in individuals from the same family sharing molecular features.

**Conclusions:** We show evidence that CRC tumors arising in HNPCC families without MMR alterations have distinctive molecular features. Overall, our work shows that systematic analysis of somatic alterations in a well-defined subset of CRCs is a good approach to provide new insights into the mechanisms of colorectal carcinogenesis.

Approximately 5% of all colorectal cancers (CRC) are diagnosed in the context of known Mendelian syndromes, principally, hereditary nonpolyposis colon cancer (HNPCC) and familial adenomatous polyposis. When considering HNPCC as a syndrome linked to mismatch repair (MMR) gene mutations, the frequency range is from 0.3% to 3% of the total CRC burden (1). Tumors from patients with MMR mutations frequently show high – microsatellite instability (MSI-H; ref. 2). The MSI-H phenotype is also found in 10% to 15% of sporadic CRCs, although these tumors differ from their hereditary counterparts at the molecular level (3, 4). For instance, the MMR deficiency seen in sporadic tumors is mainly due to *hMLH1* promoter hypermethylation. Apart from MSI, less well-characterized genetic instability phenotypes may contribute to colorectal carcinogenesis. For example, epigenetic silencing of methylguanine methyltransferase (*MGMT*), leading to an excess of G > A transitions, is a common event (5).

Early genetic events in colorectal carcinogenesis involve the deregulation of the WNT and *RAS*/*RAF*/*MAPK* pathways. The mutation profile of a particular tumor is related to the underlying phenotype instability. For example, in MSI-H tumors, WNT and MAPK pathway alterations show a shift from *APC* to *CTNNb1* and *KRAS* to *BRAF* involvement, respectively (6, 7). Although genes with repetitive sequences (e.g., *CTNNb1*) are mutation targets in tumors with a defective MMR system, mutations in nonrepetitive sequences also occur in MSI-H tumors. For example, *BRAF* V600E mutations are more common in MSI-H than in microsatellite-stable (MSS) tumors (35% versus 6%; refs. 7–9).

Up to 50% of HNPCC families meeting the stringent Amsterdam I criteria (10) do not show any evidence of mutator pathway abnormalities. Tumors arising in these families have distinctive clinical features. We hypothesize that at the molecular level these tumors may show distinctive molecular

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changes in common carcinogenic pathways (11 – 13). To test our hypothesis, we did a comprehensive study of *KRAS*, *BRAF*, *APC*, *MGMT*, and *β-catenin* somatic alterations in a well-defined cohort of 57 MMR proficiency HNPCC CRCs. As shown here, detailed tracing of molecular events in such special cohorts may unravel novel pathways to colorectal carcinogenesis.

## Materials and Methods

***Tumor specimens.*** During the period 1998 to 2006, 394 families with a clinical history suggestive of HNPCC syndrome attended the Familial Cancer Clinic at the Hospital Clínico San Carlos (Madrid, Spain). Of these families, 264 were selected for genetic testing of the *MMR* genes according to the Amsterdam I/II (10, 14) or Bethesda criteria (15). Germ line mutations were detected in 56 families, whereas the remaining 208 were considered negative. Subsequently, MSI and immunohistochemical analysis showed a strong correlation of MSI-H and negative immunohistochemical staining with the presence of a pathogenic MMR mutation (16). For the present study, we selected 37 negative families in which tumors showed MSS phenotype and positive expression of MMR proteins. These families fulfilled the following strict clinical criteria:

1. Amsterdam I/II (10, 14): 17 families (28 tumors).
2. Amsterdam borderline (families fulfilling all Amsterdam I/II criteria with the exception that the earliest age of diagnosis is allowed to be until 55 years old): 17 families (19 tumors).
3. Two CRCs diagnosed before 50 years of age in first-degree relatives: three families (four tumors).
4. One CRC and one first-degree relative with an HNPCC-related cancer. At least one cancer diagnosed under 40 years of age: three families (three tumors).
5. One CRC diagnosed ≤31 years of age without family history: three families (three tumors).

We retrieved archival pathologic specimens from 57 patients with CRC (38 females and 19 males) belonging to 37 families. Clinical records and pathologic reports were available in all cases. The average age of diagnosis was 52. We classified colon tumors as right-sided if they were located in the ascending hepatic flexure or transverse colon (22 cases), and left-sided if located in the splenic flexure, descending, sigmoid, or rectum (35 cases).

***DNA isolation.*** Genomic DNA was isolated from peripheral blood lymphocytes according to the salting out procedure (17). Tumor areas were selected on the basis of a H&E-stained slide, using the Chelex extraction method (18). Tumor DNA was isolated from the paraffin-embedded material by taking tissue punches (diameter, 0.6 mm) with a tissue microarrayer (Beecher Instruments).

***Genetic testing.*** Index cases from the 37 HNPCC families included in this study had been previously screened and were negative for the presence of germ line mutations in *MMR* genes. Mutation scanning included the analysis of all coding sequences and intron/exon boundaries of *hMLH1*, *hMSH2*, and *hMSH6* by denaturing gradient gel electrophoresis, as previously reported (19, 20). In addition, the presence of genomic rearrangements at the *hMLH1*, *hMSH2*, *hMSH6*, and *hPMS2* loci was tested by multiplex ligation – dependent probe amplification with P003 and P008 MRC-Kit, according to the instructions of the supplier (MRC-Holland).

***Microsatellite instability.*** MSI analysis was done in all paired tumor-normal tissue DNA samples testing the Bethesda panel of five markers (D2S123, D5S346, D17S250, BAT 25, and BAT 26). PCR amplifications were done with the HNPCC Microsatellite Instability Kit (Roche Diagnostic) according to the instructions of the supplier (Roche Diagnostic). Products were analyzed in an ABI310 genetic analyzer. Tumors were classified as MSS if none or just one of the five Bethesda markers showed instability.

***Tissue microarray.*** A tissue microarray was assembled from formalin-fixed paraffin-embedded tissues as previously described (21). In brief, triplicate 0.6-mm diameter tissue cores selected by a pathologist (H. Morreau) were taken from each tumor (Beecher Instruments), and subsequently arrayed on a recipient paraffin block, using standard procedures.

***Immunohistochemistry.*** Sections from the constructed tissue arrays were mounted on silanized slides (Dako). These slides were used to analyze the expression of MLH1, MSH2, MSH6, PMS2, and β-catenin. If the staining was not easily scored, immunohistochemistry was done on whole tumor sections. Both tissue microarray and whole tissue slides were stained with antibodies against MLH1 (clone G168-728, 1:50; BD Biosciences), PMS2 (clone A16-4, 1:50; BD Biosciences), MSH2 (clone GB-12, 1:100; Oncogene Research Products), MSH6 (clone 44, 1:400; BD Biosciences), and anti – β-catenin (clone 17C2, dilution 1:25; Novocastra Laboratories, Ltd.) in a DAKO TechMate 500+ automated tissue stainer, using standard protocols and procedures as indicated by the manufacturer (18).

Staining of MGMT protein was done with anti-MGMT (clone MT3.1, dilution 1:25; Neomarkers) by a manual procedure. The protocol is available upon request. Reliable immunohistochemistry data regarding MGMT and β-catenin protein expression were obtained in 44 out of 57 tumors.

Expression of MLH1, MSH2, MSH6, PMS2, and MGMT were scored as positive (+) when nuclear staining was observed in at least some tumor cells and negative (-) if the staining was observed in the internal control but not in the tumor cells. β-Catenin expression was considered aberrant if there was nuclear staining in >10% of tumor cells. All cases reported as membranous showed 0% nuclear staining except for four cases that also showed nuclear staining in >10% of tumor cells.

***KRAS and BRAF mutation analysis.*** Four primers were used to perform a nested PCR which specifically amplifies a 114 bp fragment of *KRAS* exon 2, including codons 12 and 13. *BRAF* exon 15 was directly amplified with flanking primers. All primer sequences and amplification protocols have been previously described by Brink et al. (22) and Xu et al. (23), respectively. PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced with the fluorescent Big-Days Terminators Sequencing Kit (Applied Biosystems) at Base Clear LABSERVICES.

***APC mutation analysis.*** The mutation cluster region (MCR) was amplified with primer sets: S1 (173 bp), S2 (211 bp), S3 (214 bp), and S4 (206 bp), as previously reported by Lüchtenborg et al. (24). PCR products were purified with Montáge PCRμ96 plate (Millipore). Sequencing analysis of PCR products was done at the Leiden Genome Analysis Center and analyzed with Chromas 1.5. *APC* mutation analyses were not done in 5 out of the 57 samples due to shortage of tumor DNA.

***Methylation-specific PCR of MGMT.*** Modification of DNA was done with the EZ DNA Methylation Kit (Zymo Research) according to the instructions of the supplier. Primers used for the methylation-specific PCR were previously described by Esteller et al. (25).

***Statistical analyses.*** Statistical significance for differences between groups (*P* value) was determined using Fisher's exact test, $\chi^2$, or *t* test as appropriate. All reported *P* values were two-sided, and values <0.05 were considered significant.

## Results

We investigated the incidence of somatic mutations in *KRAS* exon 2, *BRAF* exon 15, and *APC* MCR in 57 MMR proficiency colorectal adenocarcinomas arising in 37 HNPCC families (see Materials and Methods).

Overall, KRAS mutations were detected in 40.3% (23 of 57), *BRAF* mutations in 3.5% (2 of 57), and *APC* mutations in 19.2% (10 of 52) of the tumors. Similar figures are observed by restricting the analysis to index cases: 40.5% in *KRAS*

**Table 1.** *KRAS* mutation profile in HNPCC-MSS CRC cases

| Nucleotide change | Amino acid change | No. (%) [*N* = 23] |
|---|---|---|
| GGT to GAT | G12D | 9 (39.1) |
| GGT to GTT | G12V | 5 (21.7) |
| GGT to GCT | G12A | 3 (13.0) |
| GGT to AGT | G12S | 1 (4.3) |
| GGT to TTT | G12F or G12C/V* | 1 (4.3) |
| GGC to GAC | G13D | 4 (17.4) |

*We cannot distinguish if the observed change represents a novel mutation or the presence of two clonal populations.

(15 of 37), 5.4% in *BRAF* (2 of 37), and 20.6% in *APC* (7 of 34). Such results indicate that incidences are not biased by familiar clustering. Concomitant *KRAS* and *BRAF* mutations were not observed in any tumor. Six different *KRAS* mutations were observed in the present study (Table 1). Mutation G12D was found in 39% of tumors. Also, one tumor presented two mutations in codon 12. These data may be interpreted either as a novel doublet mutation, $KRAS^{G12F}$, or as evidence of two different clonal populations with $KRAS^{G12V}$ and $KRAS^{G12C}$ mutations (26). The frequency of *KRAS* substitutions was higher in codon 12 than in codon 13 (83.3% versus 16.7%). Twenty-two out of 24 DNA changes occurred at the second base of codons 12 and 13. G > A transitions accounted for 58.3% (14 of 24) of all DNA changes, whereas G > T transversions were observed in 29.2% (7 of 24). The remaining 12.5% of mutations (3 of 24) were G > C transversions. *KRAS* G > A mutations are associated with *MGMT* epigenetic silencing in sporadic CRC lesions (5). We investigated if such associations were also observed in HNPCC-MSS tumors. For this purpose, immunohistochemical analysis of MGMT expression was obtained in 44 tumors. We found a lack of expression in 6 out of 14 tumors with *KRAS* G > A transitions, and 2 out of 30 without (43% versus 7%, $P = 0.008$). We also analyzed *MGMT* promoter methylation status in those tumors with defective protein expression, observing hypermethylation in all cases (Figs. 1A and 2).

*APC* mutations were found only in 20% of the HNPCC-MSS tumors. Small insertions/deletions in repetitive sequences accounted for most *APC* mutations, although a 38 bp deletion and two substitutions were also observed (Table 2). Somatic missense mutations were detected in two tumors (V1323I and S1360F). β-Catenin was studied in 44 out of 57 HNPCC-MSS tumors (12 with *APC* mutations and 32 without). Sixteen tumors showed nuclear β-catenin, indicating WNT signaling deregulation (nine with *APC* mutations and seven without; $P = 0.003$). β-Catenin membranous expression pattern was found in the remaining 28 tumors. Curiously, three of them had a truncating mutation in *APC* (Table 2; Figs. 1B and 2).

Overall, the incidence of alterations detected in our study did not differ according to HNPCC clinical criteria, gender, site, or

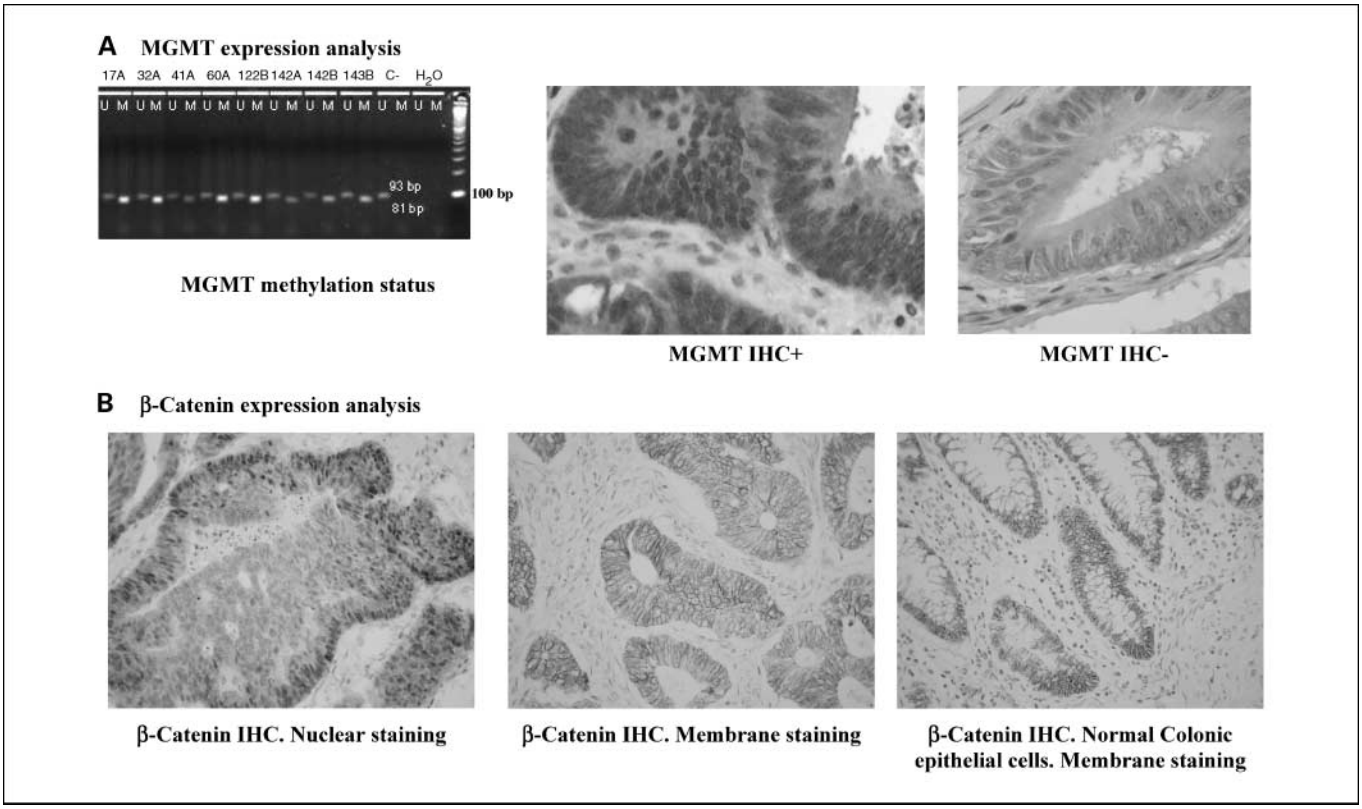


**Fig. 1.** MGMTand β-catenin expression analysis. *A,* methylation status of the MGMT promoter in eight colorectal tumors with defective MGMTexpression (*left*). PCR product indicating an unmethylated (*U lanes*) or methylated (*M lanes*) promoter. C-, control for unmethylated MGMT (positive expression of MGMT by immunohistochemistry). Right, examples of CRC cells staining positive and negative for MGMT by immunohistochemistry (*IHC*). *B,* representative examples of β-catenin nuclear (*left*), and membranous (*right*) staining.

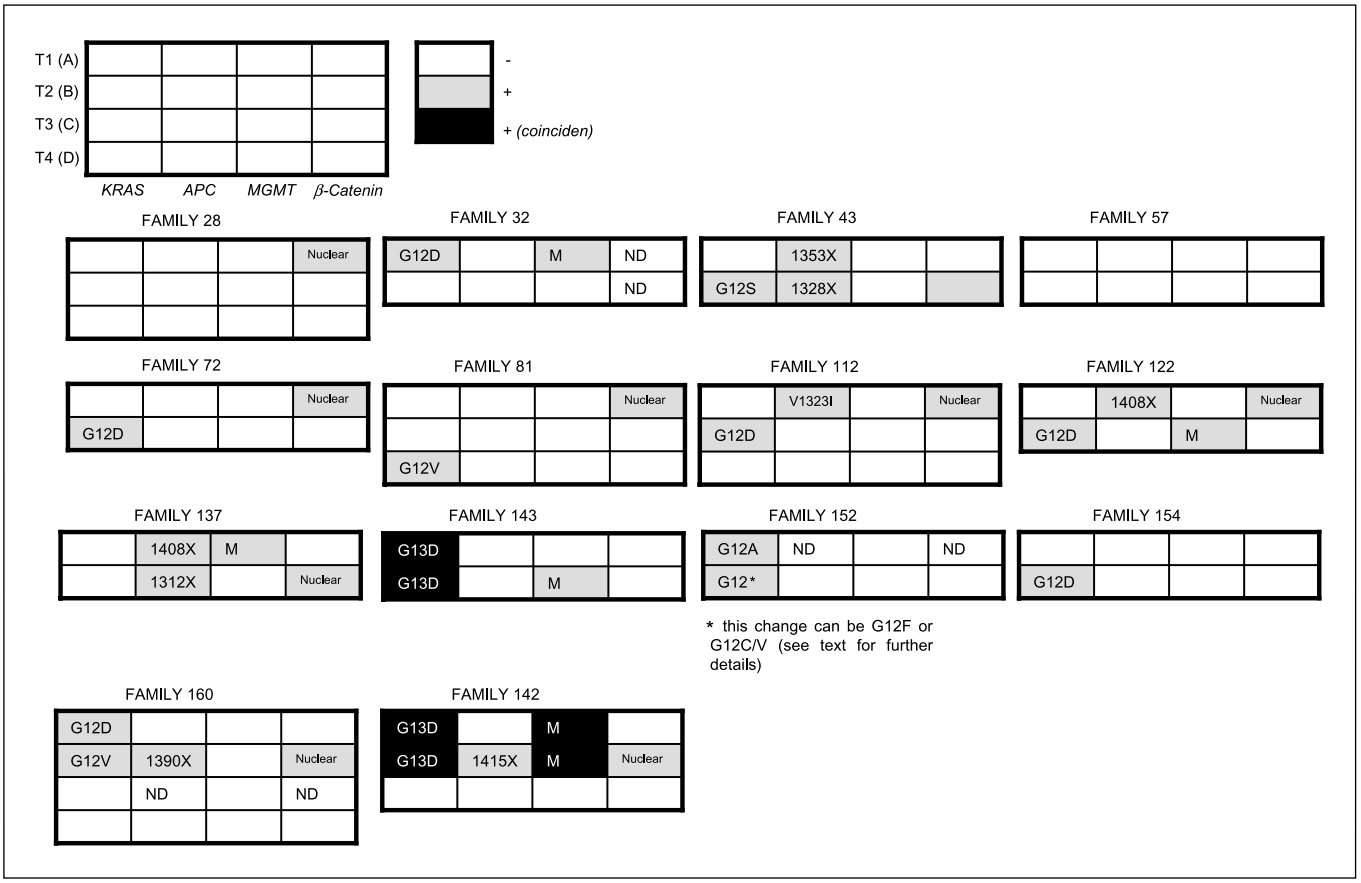


**Fig. 2.** Somatic alterations in CRC tumors clustered by family. Each panel represents a family with at least two different tumors analyzed. Rows, tumors; columns, somatic alterations. Empty boxes, lack of genetic alterations; gray boxes, presence of alteration (somatic mutation, MGMT promoter methylation, or β-catenin nuclear staining); black boxes, tumors with identical alterations. M, MGMT promoter methylation; ND, not determined.

Duke's stage (Table 3). However, it should be pointed out that all *KRAS* mutations targeting codon 13 were detected in tumors diagnosed in Amsterdam I families. In addition, both tumors with *BRAF* mutation (V600E) were right-sided and invasive (one Duke's stage C and one Duke's stage D). Interestingly, the average age of diagnosis was 10 years older in tumors showing *KRAS* mutations (58.4 years versus 47.7 years, $P = 0.001$). By contrast, the average age of diagnosis was independent of *APC* mutation status. Both tumors with *BRAF* mutation (V600E) were diagnosed at early ages (43 years). Prompted by these observations, we analyzed somatic alterations in relation to age of diagnosis. For this purpose, we compared tumors diagnosed

**Table 2.** Mutations found at the *APC* MCR in HNPCC-MSS tumors

| Tumor ID | APC Somatic mutation* | | β-Catenin immunohistochemistry | KRAS somatic status |
|---|---|---|---|---|
| T159A | 3954 del TAAT | 1303× | Nucleus | WT |
| T160B | G3982T | 1309× | Nucleus | GTT GGC (G12V) |
| T108A | 3983 del AAAGA | 1312× | Nucleus | WT |
| T137B | 3982 del AAAAG | 1312× | Nucleus | WT |
| T43B | 4034 del 38 bp | 1328× | Nucleus | AGT GGC (G12S) |
| T43A | 4091 del AA | 1353× | Membrane | WT |
| T122A | 4246 del GA | 1408× | Nucleus | WT |
| T137A | 4249 del AG | 1408× | Membrane | WT |
| T142B | 4279 ins TG | 1415× | Nucleus | GGT GAC (G13D) |
| T17A | T4520G | 1488× | Membrane | WT |
| T98B | S 1360 F | | Nucleus | GTTGGC (G12V) |
| T112A | V 1323 I | | Nucleus | WT |

*According to Genbank accession no. NM_000038.

**Table 3.** Mutation rate by clinical criteria, sex, site, and stage

| | Clinical criteria | | Sex | | Tumor site | | Duke's stage | |
|---|---|---|---|---|---|---|---|---|
| | Amsterdam I/II (%) | No Amsterdam (%) | Female (%) | Male (%) | Left (%) | Right (%) | A + B (%) | C + D (%) |
| *KRAS* | 10/28 (35.7) | 13/29 (44.8) | 15/38 (39.5) | 8/19 (42.1) | 15/35 (42.9) | 9/22 (36.4) | 13/29 (48.3) | 9/28 (32.1) |
| *BRAF* | 1/28 (3.6) | 1/29 (3.4) | 1/38 (2.6) | 1/19 (5.3) | 0 | 2/22 (9.1) | 0 | 2/28 (7.1) |
| *APC** | 5/28 (17.9) | 5/24 (20.8) | 7/35 (20.0) | 3/17 (17.6) | 7/32 (21.9) | 3/20 (15.0) | 6/24 (25.0) | 4/28 (14.3) |
| *MGMT* † | 5/25 (20) | 3/19 (15.8) | 5/33 (15.1) | 3/11 (27.3) | 5/29 (17.2) | 3/15 (20.0) | 6/20 (30.0) | 2/24 (8.3) |

*APC mutation analysis was not done in 5 out of 57 samples due to shortage of tumoral DNA.
† Reliable immunohistochemical data regarding MGMT protein expression was obtained in 44 out of 57 tumors.

above and below 50 years old (median age of diagnosis in our series). Data are shown in Table 4. Activating mutations at the *RAS/RAF* pathway were very frequent in older patients (>50 years old), and with no exception, targeted *KRAS*. In sharp contrast, mutations were less common in younger patients (≤50 years old), but similar contributions of *KRAS* and *BRAF* mutations were observed. A low percentage of *APC* mutations were detected in both groups. Lack of MGMT expression was only observed in older patients (tumors diagnosed at >50 years old), as expected in an epigenetic-related process. Interestingly, despite normal expression of MGMT, four out of five *KRAS* mutations detected in younger patients (tumors diagnosed ≤50 years old) were G > A transitions (Table 4).

Finally, it is noteworthy that we scarcely observed any degree of somatic alterations overlapping in tumors diagnosed in individuals from a single family. Data are summarized in Fig. 2. Although family 43 showed *APC* mutations in two tumors, they were of different nature (2 and 38 bp deletions). Two tumors with similar *APC* mutations (2 and 5 bp deletions) were observed in family 137. Family 152 showed different *KRAS* mutations in two tumors. Distinct tumors with identical mutation in *KRAS* (G13D) were observed in families 142 and 143. In family 142, mutations were associated with a lack of MGMT expression.

## Discussion

Frequently, the term HNPCC is used synonymously with hereditary DNA MMR deficiency. However, HNPCC is a clinical term referring to pedigree characteristics (10). Indeed, half of the families fulfilling the Amsterdam criteria do not show a MMR genetic deficiency (27). There is growing evidence that HNPCC syndromes not related to defective *MMR* genes should be considered as a different clinical entity (28). To what extent CRC tumors arising in these families follow carcinogenic pathways similar to those in MMR deficiency syndromes, is unknown. In order to investigate this issue, the present study aimed to characterize somatic alterations in HNPCC-MSS CRCs. We analyzed *KRAS*, *BRAF*, *APC*, *MGMT*, and *β-catenin* alterations.

In our subset of tumors, we found a *KRAS* mutation rate (40.3%) similar to HNPCC-MSI-H (40%; ref. 7) and sporadic-MSS CRC tumors (45%; ref. 29). In contrast, a recent study found a lower rate of *KRAS* mutations (17%) in a short cohort ($n$ = 23) of HNPCC-MSS tumors (13). According to our data, the clustering of mutations observed in codon 12 (83.3%) clearly distinguishes these tumors from HNPCC-MSI-H, in which mutations were equally distributed among codons 12 and 13. In fact, the mutation profile of G > A transitions observed in 58.3% of the tumors, clustering of mutations in codon 12 (83.3%), and G12D as the most common change, resembled those of the sporadic CRC (7). Moreover, the rate of G > T transversions (29.2%) matched that observed in sporadic-MSS and differed from sporadic-MSI-H (14%).

*KRAS* mutations have not been associated with clinicopathologic features in a large cohort of sporadic CRCs (30). Similarly, we did not find any association between *KRAS* mutations and gender, tumor site, Duke's stage, or HNPCC clinical criteria. Recently, a population-based study published a very low *KRAS* mutation rate (6%) in early onset sporadic CRC (<45 years old; ref. 31). In this regard, we observed a strong association of KRAS mutation with older age of diagnosis.

The *BRAF* mutation rate (3.5%) did not differ from that described previously in sporadic-MSS (29) and HNPCC-MSS CRC tumors (13). In sporadic CRC, *BRAF* mutations were associated with a less invasive behavior and later onset (32). In our study, the two tumors harboring *BRAF* mutations alone were invasive and early onset. This discrepancy may be explained by the fact that in sporadic CRCs, *BRAF* is strongly correlated with epigenetic silencing of *hMLH1*, an event associated with older ages of diagnosis and long-term better prognosis. Nonetheless, concomitant *BRAF* and *KRAS* mutations have been associated with lymph node metastasis (33). We cannot exclude that concomitant with *BRAF* mutations, other alterations at the *RAS/RAF/MAPK* pathway could be present in our tumors.

**Table 4.** *KRAS/BRAF/APC* mutations, lack of expression of MGMT, and age at diagnosis

| Somatic alteration | ≤50 y (%) | >50 y (%) | *P* |
|---|---|---|---|
| KRAS mutations | 5/27 (18.5) | 18/30 (60.0) | 0.001 |
| G > A | 4/5 | 10/18 | |
| G > T | 1/5 | 5/18 | |
| G > C | 0/5 | 3/18 | |
| BRAF mutations | 2/27 (7.4) | 0/30 | Not significant |
| KRAS/BRAF | 7/27 (26.0) | 18/30 (60.0) | 0.009 |
| APC mutations* | 4/25 (16.0) | 6/27 (22.0) | Not significant |
| MGMT abrogation † | 0/22 | 8/22 (36.4) | 0.001 ‡ |

*APC mutation analysis was not done in 5 out of 57 samples due to shortage of tumoral DNA.
† Reliable immunohistochemical data regarding MGMT protein expression was obtained in 44 out of 57 tumors.
‡ Fisher's exact test.

In brief, oncogenic alterations selected by sporadic and HNPCC-MSS CRC tumors at the *RAS/RAF* signaling pathway are strikingly similar, suggesting that genetic factors contributing to cancer susceptibility in HNPCC-MSS families (if any) do not select for particular alterations in this pathway.

In sporadic CRC, G > A transitions have been associated with epigenetic silencing of the *MGMT* gene (5). However, a nonmethylated signal was observed in many tumor samples of that study. Whether it represented partial gene inactivation or normal tissue contamination was not clear (5). Consequently, the study did not clarify if *MGMT* haploinsufficiency or complete lack of expression was necessary for an increased G > A transition phenotype. To avoid this uncertainty, we assessed the relationship between MGMT-defective expression and G > A transitions in HNPCC-MSS tumors by immunohistochemical analysis. With this approach, we confirmed that an association between MGMT abrogation and increased G > A transitions was also present in HNPCC-MSS tumors. MGMT abrogation was associated with older ages of diagnosis, suggesting that epigenetic mechanisms were involved. In fact, we detected aberrant promoter methylation in all tumors lacking MGMT expression, indicating that in HNPCC-MSS tumors, as previously described in sporadic CRC, promoter hypermethylation is involved in *MGMT* gene silencing.

Although *MGMT* defects are associated with somatic G > A transitions in older patients, the highest frequency of transitions was observed in younger patients, pointing to mechanisms other than MGMT abrogation inducing nucleotide instability in these patients. In turn, this suggests that *KRAS* G > A transitions are not a mere by-product of *MGMT* defects in older patients, but a change actively selected by CRC tumors.

As we have already discussed, alterations at the *RAS/RAF/MAPK* pathway suggest similarities between HNPCC-MSS and sporadic-MSS tumors. Indeed, age stratification analysis indicates that the pattern of alterations in tumors developing at age >50 are strikingly similar to sporadic cases. For that reason, we suggest that some HNPCC-MSS families were classified as such due to chance aggregation of sporadic cases.

The overwhelming majority of CRCs display active WNT signaling due to either *APC* or *CTNNB1* mutations (13). In this respect, *APC* mutation rate in our cohort of HNPCC-MSS (19.2%) was low compared with sporadic-MSS CRC (34-70%; refs. 24, 34), but was similar to HNPCC-MSI-H (21-27%; refs. 6, 34). Surprisingly, in HNPCC-MSS tumors, small deletions/insertions in repetitive sequences account for 70% of mutations, mimicking the mutation profile of HNPCC-MSI-H (34, 35). The data indicate that factors other than MMR deficiency might account for this mutation profile.

The use of immunohistochemistry to analyze β-catenin subcellular localization has been proven successful in detecting nuclear β-catenin as a marker for WNT signaling deregulation. The frequencies we found for β-catenin alterations in our HNPCC-MSS families were compatible with a recent report (13). Membranous β-catenin was found in 28 of 44 tumors, indicating inactive WNT signaling. Nuclear β-catenin was found in 16 of 44 tumors, indicating WNT signaling deregulation. Nine of these 16 tumors could be explained by mutations in the MCR-*APC* region. The other cases could be explained by APC mutations outside the MCR or by other genes like *CTNNB1* (6, 36). In any case, the β-catenin immunohistochemical analysis confirms that deregulation of the WNT pathway is not a common carcinogenic mechanism in this subgroup of tumors.

Briefly, the main conclusions of our study are that (*a*) in HNPCC-MSS CRCs the pattern of *KRAS/BRAF* mutations mimics sporadic-MSS CRCs, whereas *APC* alteration resembles those found in HNPCC-MSI-H tumors, (*b*) *KRAS* mutations are associated with older age of diagnosis, (*c*) *KRAS* G > A transitions are not a by-product of MGMT silencing, but are actively selected by tumors, and (*d*) the WNT pathway is not a common carcinogenic mechanism in this subgroup of tumors. Overall, our work shows that systematic analysis of somatic alterations in a well-defined subset of CRCs is a good approach to provide new insights into the mechanisms of colorectal carcinogenesis.

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