Detection of APC Gene Deletions Using Quantitative Multiplex PCR of Short Fluorescent Fragments

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BACKGROUND: Approximately 20% of classic familial adenomatous polyposis (FAP) cases and 70% to 80% of attenuated FAP (AFAP) cases are negative for the APC/ MUTYH point mutation. Quantitative multiplex PCR of short fluorescent fragments (QMPSF), a technique for detecting copy number alterations, has been successfully applied to several cancer syndrome genes. We used QMPSF for the APC gene to screen FAP APC/ MUTYH mutation-negative families to improve their diagnostic surveillance.

METHODS: We set up and validated APC-gene QMPSF using 23 negative and 1 positive control and examined 45 (13 FAP and 32 AFAP) unrelated members of APC/ MUTYH mutation-negative families for copy number alterations. We confirmed the results using multiplex ligation-dependent probe amplification (MLPA). We used different approaches such as sequencing, quantitative real-time PCR (QRT-PCR), and fluorescence in situ hybridization (FISH) to further characterize the identified deletions.

RESULTS: APC QMPSF was capable of detecting deletions with an acceptable variability, as shown by mean values (SD) of allele dosage for the deleted control obtained from intra- and interexperimental replicates [0.52 (0.05) and 0.45 (0.10)]. We detected 3 gross deletions in 13 (23%) of the classic FAP cases analyzed (1 complete gene deletion and 2 partial deletions encompassing exons 9 and 10 and exons 11–15, respectively). No rearrangements were detected in the 32 AFAP cases.

CONCLUSIONS: QMPSF is able to detect rearrangements of the APC gene. Our findings highlight the importance of using a copy number alteration methodology as a first step in the routine genetic testing of FAP families in the clinical setting.

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Classic familial adenomatous polyposis (FAP) is an autosomal dominant inherited disease caused by germ-line mutations in the adenomatosis polyposis coli (APC) tumor suppressor gene. FAP patients typically develop hundreds to thousands of adenomas in the colon and rectum by the third decade of life. Truncating germ-line mutations in the APC gene are responsible for the majority of FAP cases, whereas a minority of cases are caused by missense mutations. In attenuated FAP (AFAP), patients generally present with a lower number (<100) of colorectal adenomas by their fourth decade. AFAP is mainly associated with germline mutations at the 5’ and 3’ ends of the APC gene. In some cases, AFAP is associated with biallelic germline mutations in the base-excision repair gene MUTYH showing an autosomal recessive pattern. We have observed that up to 20% of the cases of classic FAP and 70% to 80% of AFAP are identified as APC/MUTYH mutation-negative by different point mutation detection strategies. Failure to detect APC germline mutations might be caused by the methodological limitations of routine diagnostic techniques, such as the masking of genomic rearrangements by the

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Nonstandard abbreviations: FAP, familial adenomatous polyposis; AFAP, attenuated FAP; wt, wild type; FISH, fluorescence in situ hybridization; QRT-PCR, quantitative real-time PCR; MLPA, multiplex ligation-dependent probe amplification; QMPSF, quantitative multiplex PCR of short fluorescent fragments; HNPCC, hereditary nonpolyposis colorectal cancer.

1 Human genes: APC, adenomatosis polyposis coli; MUTYH (or MYH), mutY homolog (E. coli); BRCA, breast cancer; MSH2, mutS homolog 2, colon cancer, nonpolyposis type 2 (E. coli); HMB5, hydroxymethylbilane synthase (formerly PBGD); REEP5, receptor accessory protein 5 (formerly CSorf18); DCP2, DCP2 decapping enzyme homolog (S. cerevisiae).
presence of the wild-type (wt) allele in the heterogeneous state.

Cytogenetically detectable interstitial deletions at 5q22 have been reported in FAP patients exhibiting some degree of mental retardation and dysmorphism (6). Submicroscopic deletions have also been detected in 6% to 17% of FAP families (6) by Southern blot analysis, apparent nonparental segregation of intragenic or flanking polymorphic marker alleles, fluorescence in situ hybridization (FISH) analysis, quantitative real-time PCR (QRT-PCR), comparative genomic hybridization, RNA analysis, and multiplex ligation-dependent probe amplification (MLPA) (6–11).

A simple, semiquantitative procedure based on quantitative multiplex PCR of short fluorescent fragments (QMPSF) has been shown to be useful for detecting genomic deletions or duplications of mismatch repair and BRCA genes (12–14). We adapted this technique for the study of copy number alterations in the APC gene in a series of unrelated FAP patients with no previously detected point mutation of the APC or MUTYH genes. We compared the results to those obtained with other techniques for determining the relative copy number of DNA sequences such as MLPA (15) and characterized the molecular nature of the alterations.

Materials and Methods

PATIENTS

Between 1999 and 2007, 121 FAP families (75 classic and 46 AFAP) were referred to our Genetic Counseling Unit. Semiautomated sequencing of the entire coding region of APC and analysis of the 2 most prevalent MUTYH gene mutations (G382D and Y165C) revealed pathogenic mutations in the APC or MUTYH genes in 76 families (62 classic and 14 AFAP). We selected 45 (13 classic and 32 AFAP) unrelated affected members of APC/MUTYH mutation-negative families for a study of possible gross APC gene rearrangements. We used DNA samples obtained from peripheral blood lymphocytes of 23 hereditary nonpolyposis colorectal cancer (HNPPCC) patients as negative controls, since a pathogenic mutation in MSH2 or MLH1 had already been found. As a positive control, we used a FAP case with a cytogenetic deletion in the 5q region associated with mental retardation. All control DNA samples were obtained using the same DNA extraction protocol (FlexiGene DNA kit; Qiagen). Written informed consent was obtained from all patients participating in the study, and the study protocol was approved by the Ethics Committee.

APC QMPSF

Between 8 and 10 exons can be amplified together in quantitative multiplex PCR assays (14, 16). The APC gene (≈100 kb) is composed of 14 small exons and exon 15, which covers more than three quarters of the coding sequence. Thus, 18 amplicons of between 130 and 330 bp were designed to cover the entire 8.5-kb coding region and distributed across 3 multiplex reactions (Table 1). As a negative control, we included in each reaction a fragment of a different gene in which deletion was not expected (HMBS, hydroxymethylbilane synthase at 11q23.3). Primers, designed using Primer Premier 5 (Premier Biosoft International), shared common melting temperatures of between 58 and 60 °C. At least 1 of the primers was located in the exonic region, and no primer covered polymorphic regions. All primers were chimeric and carried 10-nt extensions on their 5’ ends to homogenize annealing temperatures and relative primer concentrations (available on request). One primer from each pair was 5’-labeled with 6-FAM fluorochrome (Table 1). We set the distribution of the primers across the 3 multiplex reactions using WinMultiPLX v1.00 (BioData Ltd.). We amplified 100 ng genomic DNA in a final volume of 25 μL including 0.4–1 μmol/L primers, 200 μmol/L dNTPs, 1.5 mmol/L MgCl2, 1 U Taq polymerase (ThermoPrime Plus DNA Polymerase; ABgene), 1 μL DMSO, and 1.25 μL triethylenammonium acetate. After an initial denaturing step, samples underwent 23 cycles (10 s at 94 °C, 15 s at 50 °C, 20 s at 72 °C), which ensured that the amplification ended during the exponential phase. After the multiplex reactions, the DNA fragments were separated on an ABI Prism 3130 DNA sequencer and analyzed using GeneScan Analysis Software v3.1.2 (Applied Biosystems).

Three different methods were used to calculate allele dosage in this study: visual sample-to-control comparison, numerical sample-to-control comparison, and numerical cumulative comparison. For the visual sample-to-control comparison, we estimated allele dosage by superimposing the electropherogram of the tested sample onto the corresponding image for a control DNA sample after adjusting the vertical scale of the different amplicons. Allelic losses of one or more amplicons are represented by a 2-fold reduction in the intensity (peak heights) of an amplicon of the sample analyzed. In the numerical sample-to-control comparison, we calculated numerical normalized ratios (R) using the formula: R = (peak intensity_{amplicon x sample}/peak intensity_{amplicon x control})/(peak intensity_{amplicon ref sample}/peak intensity_{amplicon ref control}). An R value close to 0.5 (0.4–0.6) represented a 2-fold reduction. For numerical cumulative comparison, we adapted the analytical method used in MLPA (www.mrc-holland.com) to QMPSF. After normalization of all peak intensities to the intensity of the reference (HMBS) peak, we obtained the numerical normalized ratio (R’) of a given exon and sample using the formula R’ = normalized
peak intensity of analyzed sample/mean of normalized peak intensities of all samples. As above, R/H11032 values close to 1 and 0.5 indicate whole and half allele dosage, respectively.

MLPA ANALYSIS
MLPA analysis, carried out using an APC-specific MLPA test (Salsa MLPA Kit P043 APC, MRC-Holland) according to the manufacturer’s instructions, used 300 ng genomic DNA. After hybridization, ligation, and amplification, we calculated the PCR product ratios by dividing each measured peak area by the sum of all peak areas for the sample. The ratio of each peak’s individual relative probe area was normalized to the mean obtained with 2 control samples. We analyzed the products using the ABI 3700 DNA sequencer and Peak Scanner Software v1.0 (Applied Biosystems).

MOLECULAR CHARACTERIZATION OF DELETIONS
We performed PCR to characterize the deletion of exons 9 and 10 (forward flanking primer 5'-ACCTAT AGTCTAAAAATACCACCT-3'; reverse 5'-CTGAGC TATCTTAAGAAATATGC-3') in a final volume of 50 μL containing 0.2 μmol/L each primer, 1 U Taq polymerase (Thermoprime Plus DNA Polymerase; ABgene), 200 μmol/L dNTPs, and 1.5 mmol/L MgCl₂. After denaturation at 94 °C for 10 min, 30 cycles of PCR were performed (94 °C for 30 s; 55 °C for 30 s; 72 °C for 5 min). After using the PCR Product Purification Spin Kit/250 (Genomed), 1 μL of the amplified product was sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer’s instructions. The primers used for sequencing the breakpoint were 5’-TTTC CGGTTTCTAATCTGCTTCT-3’ as the forward primer.

### Table 1. Primers used for the APC gene QMPSF.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Size of the amplicon, bp</th>
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</tr>
<tr>
<td>1</td>
<td>TTGTCTCTTTACCCCTTTCTTTTA</td>
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<tr>
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<td>AAAGTCAAGTTTGTCAAAAGGCAATTC</td>
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Forward and reverse primers contained a special extension at their 5’ end, the sequence of which is available on request. Amplicon size includes the 5’ special extension.

⁺ Primers labeled with 6-FAM.

b Control amplicon corresponding to HMBS hydroxymethylbilane synthase (11q23) used as an internal control in all multiplex assays.
and 5′-CCCTTTTAGCAGTTCTCCAC-3′ as the reverse primer.

We performed QRT-PCR with the Roche LightCycler® 2.0 System according to the SYBR Green Detection methodology. We used 8 sets of primer pairs, including a pair that amplified a fragment used as a reference for the presence of the 2 alleles (APC_e5) and a pair that amplified a deleted fragment (APC_e15) (see Supplemental Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue7). Reactions were performed in a volume of 10 μL containing 1 μL LightCycler Fast Start DNA Master SYBR Green I (Roche), 1.5 mmol/L MgCl₂, 0.5 μmol/L of each primer, and 50 ng genomic DNA for 40 cycles (95 °C for 6 s; melting temperature for 16 s; 72 °C for 20 s). The samples were then subjected to melting curve analysis from 60 to 95 °C. Crossing points (the cycle number when the amplification becomes exponential) were determined using LightCycler software. We calculated the gene copy number using the relative standard curve method (17).

We performed FISH on chromosome spreads obtained from peripheral blood of the relevant patients. Cultures were set up a few hours after blood collection in heparinized tubes. We added 500 μL whole blood to a flask containing 9 mL RPMI 1640 (Gibco) with 10% FCS, 5 mg/L phytohemagglutinin (Gibco), 100 000 U/L penicillin, and 100 mg/L streptomycin. Cultures were set up in duplicate and incubated at 37 °C for 72 h. We harvested cells after colcemid treatment (0.4 mg/L) for 20 min and then performed FISH. Briefly, we used 7 different probes from chromosome 5q22 to cytogenetically clone the deletion breakpoints RP11-809J10, RP11-619D6, RP11-616C24, RP11-389E12, and RP11-145D17 (ordered from the centromere to the telomere and covering approximately 1 Mb). All probes were obtained from the 32K BAC Rearray of the Children’s Hospital Oakland Research Institute (www.chori.org/bccpcc) and kindly provided by Luis Pérez-Jurado. Probes (1 μg of each) were directly labeled by a standard nick translation reaction, hybridized in pairs, and detected according to standard protocols (18). The FISH analysis was performed under an Olympus BX60 fluorescence microscope equipped with the appropriate filter set, and images were captured using the Cytovision software (Applied Imaging).

Results

DEVELOPMENT AND VALIDATION OF THE SEMIQUANTITATIVE MULTIPLEX PCR

To determine whether the assay was optimal for the screening of genomic copy number alterations in the subset of polyposis patients, we analyzed 23 negative controls and 1 positive control (the cytogenetical deletion at 5q). Using numerical cumulative comparison, the R’ (SD; range) of the 23 negative controls was 1.00 (0.08; 0.83–1.21) taking into account all exons and samples (Fig. 1A). Most of the outlier values within the 23 control intensities belonged to a single control. The positive control showed an R’ of 0.55 (0.06; 0.47–0.67) (Fig. 1A). Using numerical sample-to-control comparison (Fig. 1B), R values for the deleted control were 0.55 (0.07; 0.39–0.77). The variability was concentrated in a few amplicons evenly distributed along the 3 multiplex PCR reactions, making it unlikely that the robustness of the technique was affected. Visual sample-to-control comparison revealed a 2-fold reduction of peak intensity when superimposing the deleted control on a negative control in all APC amplicons (see Supplemental Fig. 1 in the online Data Supplement). No reduction was observed when comparing the different negative controls (data not shown).

We assayed 10 replicates of both a negative control and the deleted control in the same experiment to assess the intraexperimental variability (Supplemental Data Fig. 2A). Using the cumulative comparison, mean R’ values of 1 (0.09; 0.73–1.25) and 0.52 (0.05; 0.38–0.71) were obtained for the normal and the deleted controls, respectively. As in the previous assay, the variability was concentrated in a few amplicons, and more extreme values appeared in only 1 or 2 amplicons of a replicate, thus reducing the likelihood of interpretation errors. Finally, interexperimental variability was studied in 13 independent QMPSF assays using the deleted control. Numerical cumulative comparison with the 23 HNPCC patients yielded R’ values of 0.45 (0.10; 0.14–0.69) (see Supplemental Fig. 2B in the online Data Supplement). There was no overlap between any of the wt and deleted control ratios.

Thus, QMPSF was able to detect the loss of 1 copy of the gene for all fragments analyzed, with an acceptable amount of intra- and interexperimental variability.

DELETION SCREENING IN PATIENTS

We used the above data to establish the following experimental design incorporating 2 duplicates of 2 wt controls in each run and a single sample of the deleted control. The run was disregarded when the ratio between replicates of wt controls was >1.2 or <0.8. Once a copy number variation was observed, the experiments were independently replicated at least twice using independent DNA aliquots. Deletions were detected in 3 of the 13 (23%) selected APC/MUTYH mutation-negative classic FAP families (see Supplemental Table 2 and Supplemental Fig. 3 in the online Data Supplement). Two of these deletions were partial, and the other encompassed the entire gene. The partial
deletions encompassed exons 9 and 10 and from exon 11 to all amplicons of exon 15, respectively. The MLPA analyses performed for the 13 classic FAP families confirmed the QMPSF results. No gross rearrangements were detected in the 32 AFAP mutation-negative families.

Regarding genotype-phenotype correlation in classic FAP families, carriers of gross deletions (n = 4/H11005, including the cytogenetically deleted control) were diagnosed at an earlier age [23.25 (7.09) years] than APC/MUTYH mutation-negative index cases [36.9 (7.84) years; n = 10; P = 0.028, Wilcoxon rank-sum test] (see Supplemental Table 2 and Supplemental Fig. 3 in the online Data Supplement). Differences were detected with respect to other APC gene mutation carriers [29.28 (13.39) years; n = 57], although the differences were not significant, possibly owing to the low number of deletion carriers. In contrast, no differences were observed in the number of colorectal polyps, the presence of extracolonic manifestations, or family history.

MOLECULAR CHARACTERIZATION OF THE PARTIAL APC DELETIONS

We confirmed all 3 of the observed deletions by replicating QMPSF assays and using MLPA. Further characterization was carried out in partial deletion carriers, which provided insight into the molecular nature of these aberrations.

In an index case, QMPSF analysis showed a deletion removing exons 9 and 10 (Fig. 2, A and B). Genomic DNA PCR encompassing exons 8–11 revealed the amplification of an abnormal fragment of approximately 4 kb in the affected patient, corresponding to the junction fragment comprising the deleted region (Fig. 2, A and C). Sequence analysis revealed that the first 3007 bp of intron 8 were fused to the last 496 bp of intron 10, deleting a 8011-bp fragment of the gene (g.80715_88725del8011; NC_000005.8; GI:51511721) (Fig. 2D). Alignment of intronic sequences using the RepeatMasker program revealed that the recombination did not involve any flanking repeat elements, suggesting a nonhomologous recombination process (Fig. 2D).

Fig. 3 shows a schematic representation of molecular studies done in an index case carrying a deletion of exon 11 to all fragments of exon 15. A QMPSF pattern showing 2-fold reduction of intensities of these exons was detected in the index case (Fig. 4A) and in another affected relative (data not shown). To further characterize the 3′ extension of the deletion, genomic DNA QRT-PCR was performed on eight fragments up to 400 kb downstream of the deleted area using exon 5 of the APC gene as a reference for normalization (Fig. 3, Fig. 4A; see Supplemental Table 1 in the online Data Supplement). Samples from an affected member and a healthy relative were compared with control DNA. The 3′ breakpoint was located within a region of approximately 83 kb encompassed by fragments REEP5_e3 and DCP2_e2 in the affected subject (Fig. 3, Fig. 4B). FISH analysis using 5 probes covering 835 kb also confirmed the approximate location of the breakpoint (Fig. 3, Fig. 4C). Probes corresponding to RP11-809F10, RP11-619D06, RP11-389E12, and RP11-145D17 clones.
yielded a double signal concordant with the QMPSF and QRT-PCR results. Although approximately half of the area covered by RP11-619D06 was deleted, the nondeleted area was sufficient to hybridize and identify the corresponding fluorescent signal. Only the RP11-616C24 probe yielded a single signal. No attempt was made to sequence the 5' breakpoint.

**Discussion**

We designed and carried out the APC QMPSF, demonstrating that it is capable of reproducibly detecting APC gene deletions. The proportion of gross deletions detected (23%) is similar to that found in previous studies (15%) analyzing well-defined FAP families with MLPA (8, 9), a common reference method used in copy number alteration studies, demonstrating that the method is a suitable complementary approach for gene dosage screening. Additionally, our results highlight the importance of performing APC deletion analysis in the assessment of classic FAP families.

Copy number variation is known to be an important factor in determining genetic predisposition to disease, in particular cancer. Germline deletions in...
cancer genes have been associated with a number of hereditary cancer syndromes including neurofibromatosis, HNPCC, hereditary breast cancer, and FAP. Duplications have been detected, albeit at a lower frequency (19). Various techniques have simplified this type of analysis: MLPA is one widely used method (15), real-time PCR methods have also been successfully developed (20), and QMPSF is a simple semiquantitative method that had already been used in the analysis of germline and somatic copy number variations (12–14, 21). Whereas primer design is a common critical step in all of these methods, the quality of DNA may also influence the final outcome. To minimize the putative impact of this source of variability, the entire procedure for sample collection and processing must be highly standardized.

In this study, APC QMPSF consistently detected the loss of 1 copy of all gene fragments. In this approach, it is not necessary to use the large number of control probes included in each MLPA reaction. On the other hand, while MLPA is performed in a single PCR reaction, QMPSF requires 3 reactions. The even distribution of exons across the 3 multiplex reactions diminishes the possibility of artifacts. In contrast with the ready-to-use MLPA kit, our in-house QMPSF approach may be more prone to variability when used in other centers. Finally, the flexibility of QMPSF allows the exploration of any genomic region of interest without the need for a specific commercial kit.

The method used to analyze allele dosage is also an important factor. We prefer the numerical cumulative approach, as it provides more accurate calculations when multiple negative samples are considered. This is often the case, since most of the tested samples will be negative. A strategy for maintaining accuracy when analyzing a smaller number of samples is to include negative controls. Alternatively, the numerical sample-to-control comparison can be used. In the case of MLPA, the latter approach is not routinely used, so it is necessary to analyze a relatively large number of samples in each assay. Although visual inspection can be more intuitive, it should always be used in combination with the sample-to-control numerical calculation.

Robustness is vital if the results of the assay will be used in the routine clinical setting. All deletions detected in the present study were confirmed by performing independent replicates of the QMPSF analysis and by MLPA, which validated the reliability of our results. Although molecular characterization of deletions is strictly necessary only when only 1 or a few exons are altered (22, 23), its importance should not be underestimated in the case of aberrations involving larger numbers of exons. Therefore, we attempted to further characterize the 2 partial deletions found. We used QRT-PCR using a relative quantification approach (relative standard curve method) and FISH analyses to obtain a partial characterization of the deletion encompassing exon 11 to the 3’ end of the gene, in which both boundaries were completely unknown. Finally, the exon 9 and 10 deletion was detected by QMPSF and carefully characterized using PCR and breakpoint sequencing. We believe that the diagnosis of gross rearrangements should not be made unless it has been confirmed by more than one analytical method.

Gross deletions were detected in 23% (3 of 13) of the APC/MUTYH mutation-negative classic FAP families analyzed. When all classic FAP families were considered, gross deletions were found in approximately 5% (4 of 75), including the cytogenetically detected deletion used as a control. This prevalence is consistent with previous reports (6, 8, 9) and corroborates that comprehensive mutation scanning of APC must include gene dosage analysis in mutation-negative classic FAP families. QMPSF is a straightforward, rapid, and low-cost screening technique—similar to MLPA—that should be used before mutational analysis of coding sequences, which is consistent with the conclusions of previous reports (24). Although other more advanced methods such as array-based comparative genomic
Fig. 4. Characterization of gross deletion involving exons 11–15 of the APC gene.
(A), QMPSF of APC showing the heterozygous deletion encompassing the region from exon 11 to the last amplicon of exon 15. Electropherograms from a control (in blue) and the carrier of the deletion (in red) were superimposed using HMBs as the internal control. (B), QRT-PCR results from analysis of fragments shown in Fig. 3 (the names are indicated in the first column of the table). The ratios shown were calculated by taking the APC_e5 fragment as the reference (ratio = 1) for a healthy (wt subject) and a mutated (deleted subject) individual compared with a control DNA sample. Ratio approximately 1 means that the fragment is not deleted and ratio approximately 0.5 indicates the deletion of the fragment. (C), All FISH probes were hybridized over deleted patient metaphases. The presence of fluorescence in only one chromosome 5 depicts the deletion of the covered region. Probes span the following regions: RP11-809J10 (111925202–112128672), RP11-619D06 (112120960–112271440), RP11-616C245 (112223260–112387360), RP11-389E12 (112408900–112557640) and RP11-145D17 (112557148–112760819). A control probe from the 5q region was also used. The magnification shows the deletion of probe RP11-616C245 (in green) in one of the homolog chromosomes 5 and the overlapping signals (RP11-616C245, in green, and RP11-389E12, in red) in the other nondeleted homolog.
hybridization may eventually provide better resolution, their current cost makes them unaffordable as a diagnostic screening tool. As in most previous research, no gross rearrangements were detected in the 30 AFAP families. Nonetheless, deletions have occasionally been associated with an attenuated phenotype (25–27), making it unwise to rule out the use of deletion screening in this setting. In the limited set of cases analyzed in this study, deletion carriers had an earlier age at diagnosis, although this association was not found in a recent review (25). As reported, extracolonic features commonly occur in these cases.

In summary, QMPSF is a robust method for APC copy number alteration analysis either as the principal screening method or as a complementary tool for confirming MLPA results. Our results also confirm the relevance of gross germline deletions in APC/MUTYH mutation-negative classic FAP families and support the use of QMPSF in the diagnostic algorithm for FAP patients. Importantly, this technique is not restricted to germline FAP/AFAP cases. It has been shown recently that the use of QMPSF to assess somatic copy number variations may be of prognostic value in colorectal cancer (21).

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