Unexplained polyposis: a challenge for geneticists, pathologists and gastroenterologists


Two main colorectal polyposis syndromes have been described, familial adenomatous polyposis and MUTYH-associated polyposis syndromes. Some polyposis remains unexplained: 20% of adenomatous polyposis and serrated polyposis. The aim of this study was to evaluate in a cohort of patients with unexplained polyposis whether a genetic defect could be detected. Individuals presenting polyposis with more than 40 adenomas or more than 20 serrated polyps (hyperplastic, sessile serrated and mixed), without causative mutation identified, were included. Complementary explorations on APC or MUTYH were performed: search for APC mosaicism, splicing-affecting mutations, large genomic rearrangement of MUTYH. Four genes of Wnt pathway (AXIN2, PPP2R1B, WIF1, SFRP1) and two genes of transforming growth factor-β (TGF-β) pathway (SMAD4, BMPR1A) were screened for germline mutation. Twenty-five patients had an unexplained adenomatous polyposis (familial or sporadic). Five pathogenic mutations were found: four in APC gene (with one case of mosaicism) and one in BMPR1A gene. The exploration of APC mosaicism was better performed from adenoma DNA with high-resolution melting. The screening of the candidate genes did not find any causative mutation. Thirteen individuals had an unexplained serrated polyposis and a frameshift on SMAD4 gene was identified. All mutations were identified in familial cases of polyposis. After new pathological examination, both BMPR1A and SMAD4 cases were found to be associated with a juvenile polyposis while the polyposis was initially described as adenomatous or undetermined. In 17% (6/38) of the patients the causative mutation of the polyposis was identified. Genetic causes were heterogeneous. Sporadic polyposis patients must be considered as potential APC mosaicism. The histological classification of polyposis is strongly important in direct genetic exploration.

Conflict of interest

No conflict of interest to report.

Two main hereditary adenomatous colorectal polyposis syndromes have been described, namely familial adenomatous polyposis (FAP) and MUTYH-associated polyposis (MAP) syndromes. FAP has an incidence at birth of about 1/8300, and accounts for less than 1% of colorectal cancer (CRC) cases. FAP is characterized by an autosomal dominant inheritance where the affected subjects...
develop hundreds to thousands of adenomatous polyps throughout the whole colon, usually during their teenage years (1). The occurrence of colon cancer is a frequent event after the second decade of life. This syndrome is also characterized by the development of a variable range of life-threatening extracolonic manifestations (upper gastrointestinal tumours, desmoid tumours and more benign manifestations like osteoma) (2). Germline mutations in the tumour suppressor gene APC are responsible for FAP and the mutation detection rate ranges above 75% (1). If not inherited, germline mutations causing genetic disorders may occur de novo in early embryogenesis and account for 10–25% of FAP (3, 4). De novo mutations may occur during cell division, leading to a mosaicism of wild-type and mutated cells in some cases. Few cases of mosaic APC mutations have been previously reported (5–7). Currently, mosaicism is estimated to be present in one fifth of de novo cases of FAP (8, 9). Bi-allelic mutations of MUTYH, responsible for adenomatous polyposis, with an increased risk of CRC, were described in 2002, and this recessively inherited polyposis is known as MAP (10). Subsequent studies have shown that patients with bi-allelic mutations of MUTYH have between 20 and 100 colorectal polyps, some duodenal and gastric polyps but exceptionally extracolonic features of FAP such as demoid and hepatoblastoma (11). MAP is responsible for about 20% of adenomatous polyposis without APC mutation identified (12).

Despite progress in understanding adenomatous polyposis, around 20% of cases remain APC and MUTYH mutation negative by conventional methods, including numerous sporadic cases. Patients without genetic explanation are considered as FAP and they as their families will undergo endoscopic screening and follow-up similar to polyposis. Different causes of unexplained polyposis are suggested such as unclassified variants identified in genomic DNA of known genes (APC, MUTYH) whose deleterious consequences remain to be determined (splicing effect, co-segregation analysis); unrecognized mutations in known genes (APC, MUTYH) by routine molecular methods; mutations in genes involved in non-adenomatous polyposis (SMAD4, BMPRIA) (13) if polyposis is misclassified. By screening different genes for germline mutations in 47 ‘multiple’ adenoma patients, Lipton et al. (14) identified putative pathogenic change in BMPRIA. Mutations of other genes might be involved such as other regulatory genes of the Wnt signalling pathway. Indeed, APC regulates Wnt signalling by controlling the levels of β-catenin reaching the cell nucleus (2).

Aside from polyposis with adenomas, serrated polyposis has been recently described and associated with a certain risk of CRC (15). The serrated polyposis includes hyperplastic polyposis syndrome (HPS), sessile serrated polyposis and mixed polyposis. HPS is a rare disease including patients with one of the following symptoms: more than 20–30 colorectal hyperplastic polyps (HP), at least one HP with a size greater than 1 cm, at least one HP in the proximal colon with a relative with HP (16). It remains poorly defined, and little is known about its genetic basis. Hereditary mixed polyposis syndrome comprises a collection of polyposis syndromes showing a mixture of various types of polyps. In some families with this syndrome, mutations in the BMPRIA gene were shown (17–19). The nosological status of the remaining cases of serrated polyposis remains unclear.

In all these mutation-negative adenomatous or serrated polyposis, a constitutive genetic or epigenetic cause is likely because the occurrence of numerous polyps cannot be satisfactorily explained by exogenous factors. The knowledge of the underlying factors has major significance for our understanding of colorectal tumourigenesis and for counselling affected families. Therefore, the aim of this study was to re-investigate the genetic origin of the disease in a series of patients with unexplained adenomatous polyposis (familial or sporadic) after routine molecular diagnosis. Additional investigations were performed in known polyposis genes (APC, MUTYH) to increase the mutation detection rate. We also completed analysis by screening genes of transforming growth factor-β (TGF-β) pathway (BMPRIA, SMAD4). Finally, we selected and screened four genes of the Wnt signalling pathway: AXIN2, PPP2R1B, WIFI, SFRP1 both of which have been implicated in colorectal tumourigenesis and have already been found mutated in somatic colorectal cells (19–22). We also searched for a genetic explanation in serrated polyposis: we screened genes of TGF-β pathway (SMAD4, BMPRIA) for germline mutations in patients with unexplained serrated polyposis.

Materials and methods

Patient selection and characteristics

Our polyposis registry included 210 patients with multiple colorectal polyposis (with more than five polyps) who came for counselling at the oncogenetic consultation. Consent for the genetic study was obtained from patients, the pedigree of the family was drawn and a blood sample was collected for DNA extraction. All patients

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were screened for *APC* germline mutations by common diagnostic methods (direct sequencing of exons and large rearrangement screening) and for *MUTYH* germline mutations by direct sequencing.

**DNA and RNA extraction**

Patients’ DNA was isolated from peripheral lymphocytes using an automated Extragene® extractor (Genomic Industry, Archamps, France) with a DNA extraction kit Wizard Genomic DNA® (Promega France, Charbonnières-les-Bains, France) according to the manufacturer’s standard protocol. Extraction from buccal swab used FTA® (WHAT-MAN, Maidstone Kent, UK). DNA extraction from frozen tissues used DNA mini Kit QiaGen® (QIAGEN, Courtaboeuf, France). Total cellular RNA was extracted with PAXgene Blood RNA purification system (QIAGEN). Quality of DNA and RNA was assessed with the Nanodrop® technology (Coleman Technologies, Orlando, FL).

**Mutation analysis**

Mutation analysis of the *APC*, *MUTYH*, *AXIN2*, *PPP2R1B*, *WIF1*, *SFRP1*, *SMAD4*, *BMPR1A* genes was performed by direct sequencing. The complete coding sequence of these genes, including exons and intronic junctions, was amplified by polymerase chain reaction (PCR). Each primers were designed with primer3 software (Frodowpitk.edu/cgi-bin/primer3/primer3_www.cgi). Primer sequences and PCR conditions are available on request. The PCR products were cleaned up on a Multi-Screen PCR 96-well plate (Millipore, Billerica, MA). Sequencing reactions were performed in forward and reverse orientations using the ABI BigDye Terminator v1.1 cycle sequencing kit (QIAGEN). The products of the sequencing reactions were cleaned up using Sephadex G-50 (GE Healthcare Life Sciences, Piscataway, NJ) in a Multi-Screen-HV 96-well filter plate then run up on an ABI 3730 DNA sequencer (Applied Biosystems, Courtaboeuf, France). The resulting sequence data were analysed with ABI Seqscape software, version 2.5 in comparison with the reference sequences of human *APC*, *MUTYH*, *BMPR1A*, *SMAD4*, *SFRP1*, *WIF1*, *PPP2R1B*, *AXIN2* genes (NM_00003814, NM_0011048171, NM_004329.2, NM_005359, NM_003012, NM_007191, NM_181699).

**Search for *APC* mosaicism**

All patients included in our polyposis registry as *de novo* cases with no known family history of FAP in the siblings or the parents were selected for potential *APC* mosaicism (*n = 17*). A second reading of *APC* sequences was carried out for these patients to look for weak signals suggesting mosaicism. In case of a suspected signal or a suggestive family history (affected offspring), we analysed tumour-derived cells extracted from frozen tissue by DNA sequencing (if frozen tissue available). The identified mosaic was analysed by high-resolution melting (HRM) analysis. The level of mutation detection was calculated by performing mutated DNA dilutions. HRM analysis of the genomic DNA samples was carried out on a LightCycler 480 System (Roche Diagnostics, Meylan, France). Reaction mixture for HRM consisted in 0.3 μM of each primer, 1X LC480 HRM Master Mix (containing fluorescent intercalant agent ResoLight dye) (Roche Diagnostics) and 20 ng of genomic DNA samples. Assays were carried out in a 96-well format in 20 μl reaction volume and were performed using the touchdown PCR cycling and HRM conditions as follows. PCRs were initiated with a 10-min hold at 95°C, followed by 42 cycles of 95°C for 10 s, a touchdown annealing step (decreasing 1°C/cycle) ranging from 65°C to 55°C for 10 s and 72°C for 20 s. Each PCR run contained one negative (no DNA template) control.

**Analysis of large genomic rearrangement for *APC*, *SMAD4* and *BMPR1A***

The screening of large deletions was performed by multiplex ligation probe amplification (MLPA) using the MLPA kit SALSA P043 for the *APC* gene and SALSA MLPA kit P158-B1 JPS for the *SMAD4* and *BMPR1A* gene (MRC Holland, Amsterdam, the Netherlands) according to the manufacturer’s instructions. Fragment analysis of multiplex PCR was carried out on the ABI 3730 DNA analyser (Applied Biosystems), using GeneMapper software, version 4.0 (Applied Biosystems).

cDNA analysis of *APC* and *MUTYH* genes

cDNA was generated using the oligoIt protocol of SuperScript™ II Invitrogen® (Invitrogen, Carlsbad, CA). PCR products were generated from the *APC* cDNA and MUTYH cDNA (primers available on request). The screening of large rearrangement in MUTYH gene was performed through cDNA analysis. Because of the small length of the coding sequence, we performed the amplification of the total cDNA (1770 bp) and looked
Table 1. Clinical features of unexplained polyposis patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Adenomatous polyposis</th>
<th>Unclassified polyposis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>Age at the time of diagnosis</td>
<td>44 years ± 16</td>
<td>51 years ± 20</td>
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<tr>
<td>More than 100 polyps</td>
<td>4 (16%)</td>
<td>3 (23%)</td>
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<tr>
<td>Familial history of polyposis</td>
<td>7 (28%)</td>
<td>5 (38%)</td>
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<tr>
<td>Familial history of CRC</td>
<td>12 (48%)</td>
<td>6 (46%)</td>
</tr>
<tr>
<td>Upper gastrointestinal polyps</td>
<td>10 (40%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Histological types of polyps</td>
<td>25 (100%)</td>
<td>9 (69%)</td>
</tr>
<tr>
<td>Adenomatous</td>
<td>25 (100%)</td>
<td>9 (69%)</td>
</tr>
<tr>
<td>Serrated</td>
<td>0 (0%)</td>
<td>6 (46%)</td>
</tr>
<tr>
<td>Hyperplastic</td>
<td>0 (0%)</td>
<td>11 (85%)</td>
</tr>
</tbody>
</table>

CRC, colorectal cancer.

for a difference in the length of the amplicon performing the migration of the PCR products on a 1% ethidium bromide-stained agarose gel. cDNA analysis was performed on the APC mRNA to study the effect of an unclassified variant (c.1549-8A>C) on splicing.

Results

Patients

Thirty-eight patients were included. We included all individuals with more than 40 adenomas (n = 25) or more than 20 polyps (i.e. serrated, hyperplastic or association of several types of polyps) (n = 13) and with no identified mutation in APC or MUTYH gene. Table 1 presents clinical features of the patients. The mean age at diagnosis was 46 (±18). Seven patients (18%) had more than 100 adenomas. A family polyposis history was present in 32% and a family history of CRC was present in 47% of the patients.

Complement analysis for APC and MUTYH genes for point mutation and large rearrangement

For three patients with few polyps and a later age at diagnosis, screening of only MUTYH was previously performed. For these patients, we completed the analysis with point mutation and large rearrangement of the APC gene. For one patient with FAP-marked phenotype, MLPA was performed for the second time and identified an exon 14 deletion that had been missed in previous experiments. The new MLPA experiment was performed using a new set of probes provided by the manufacturer.

Large rearrangement analysis was performed on cDNA for MUTYH gene (n = 6). Electrophoresis migration of cDNA MUTYH was performed for six patients with available RNA. PCR amplification from cDNA MUTYH resulted in a predicted product of 1770 bp in all samples. We did not find any large rearrangement of the MUTYH gene.

Search for APC mosaicism

The second reading of APC sequences of 17 patients with no family history, selected as potential mosaic, revealed one frameshift mutation c.3202_3205del (p.Ser1068GlyfsX57). This mutation was not a mosaic, but had been missed because of a too short overlap of the previous set of primers designed for this fragment of exon 15. Second, among the 17 patients in whom a mosaicism was searched, one case was found: a 36-year old male with mandible osteomas and multiple colorectal adenomas had a 5-year old daughter with mandible osteomas. Direct sequencing of APC in the daughter found the c.4666del (p.Thr1556LeufsX9) mutation. This deletion was not identified in the first sequencing of the proband APC gene, but we found a weak signal in the father’s sequence corresponding to mutation (Fig. 1). The low level of the mutated allele was confirmed for the proband by analysis of other tissues. Colon adenomas, normal colonic cells and cell of the oral epithelium were available. The mutation was hardly detectable, in the DNA extracted from normal colonic cells and cells of the oral epithelium but the mutation was found at a higher level in DNA extracted from adenomas compared to leucocyte DNA (Fig. 2).

The HRM method was also tested on this patient and his daughter. Several dilutions of mutated DNA in wild-type DNA showed that the HRM technique allows a 2.5% proportion of the mutated allele to be detected (Fig. 3) and was better than sequencing. HRM mutated DNA dilution analysis determined that cells with the mutated APC allele represent 2.5% in father’s leucocytes DNA.

Analysis of APC splice mutation

An APC intronic variant c.1549-8A>C had been previously identified. In silico predictions [Splice Site Finder (http://violin.genet.sickkids.on.ca/~ali/splicesitefinder.html), MaxEntScan (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html), NNsplice (http://www.fruitfly.org/seq_tools/splice.html), Gene Splicer (http://www.tigr.org/tdb/GeneSplicer/gene_spl.html)] of splicing did not support a deleterious splicing, but to address the possible molecular consequence of this intronic variant on mRNA splicing, PCR was performed
Fig. 1. Sequence analysis showing the APC mutation c.4666delA in genomic DNA from blood of the father (mosaicism) and his daughter.

Fig. 2. Comparison of sequencing results obtained from blood, normal colonic cells, oral epithelium cells and colonic adenomas.
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on the APC cDNA. Characterization of the cDNA from this mutant APC allele revealed a longer mRNA in which seven nucleotides was added to exon 12 through a cryptic acceptor splice site. This insertion created a frameshift and the introduction of a premature stop codon (Fig. 4).

Mutational analysis of two TGF-β pathway genes

Screening of SMAD4 and BMPR1A genes in adenomatous polyposis (n = 25) showed a deleterious duplication c.218dupA in BMPR1A (p.Asn73LysfsX2). Initial histology of this case reported some inflammatory polyps associated to the adenomas. A second analysis by another pathologist revealed that these polyps are compatible with juvenile polyposis. We did not find any large genomic rearrangement of SMAD4 and BMPR1A gene.

Mutational analysis of SMAD4 and BMPR1A on 13 patients with unexplained serrated polyposis showed a pathogenic mutation c.1264_1265delinsA (p.Pro422MetfsX14) in the SMAD4 gene. The first histological analysis found some unclassified polyps. A second analysis by another pathologist revealed that these polyps were compatible with juvenile polyposis.

Mutational analysis of four Wnt pathway genes

Mutation analysis of four genes involved in Wnt signalling pathway, SFRP1, WIF1, AXIN2 and...
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*PPP2R1B*, showed no deleterious mutation in the set of 25 patients. A rare variant c.1235A>G (p.Asnn412Ser) was found on the *AXIN2* gene. We tested this variant on 275 healthy control subjects with the HRM method and sequencing of variant profiles and it was identified in four healthy subjects (allele frequency = 0.72%) showing that this variant is a polymorphism.

**Discussion**

FAP and MAP syndromes are the two main causes of hereditary colorectal polyposis. About 20% of polyposis remains genetically unexplained with no mutation identified in the *APC* or *MUTYH* gene. We have defined two groups of unexplained polyposis: unexplained adenomatous polyposis and serrated unexplained polyposis (hyperplastic, sessile serrated and mixed polyposis). A combination of extensive mutation screening in *APC* and *MUTYH* genes and screening of other candidate genes for polyposis predisposition was performed in 38 unexplained polyposis patients. Six pathogenic mutations were found: four mutations in the *APC* gene: a frameshift, a large deletion, a frameshift mosaic mutation, and a splice mutation in *APC* gene with the demonstration of the pathogenic effect of the c.1549-8A>C variant, two mutations in genes of TGF-β pathway: a frameshift mutation in the *BMPR1A* gene and a frameshift mutation in the *SMAD4* gene. Overall, an additional causal mutation of polyposis was identified in 17% (6/38) of the cases selected in our study. All mutations were identified in familial cases of polyposis (even in the mosaic case with associated signs in the family) whereas 32% of included patients had familial cases of polyposis.

These results highlight the importance of complementary explorations for patients with typical polyposis. The results emphasize the need to upgrade molecular analysis of genes when the phenotype is evocative, and if significant improvements have been made on molecular method.

cDNA analysis is important for the molecular diagnosis of adenomatous polyposis, in particularly for assessing the functionality of rare variant identified in genomic DNA. Several cases of splice variants have already been described for *APC* and *MUTYH* (20–23). We showed, in our study, that *APC* intronic variant c.1549-8A>C is pathogenic even if *in silico* splice sites are unable to predict deleterious effect.

Mosaicism might underlie a fraction of sporadic FAP cases. It is estimated that 10–20% of de novo cases of FAP are mosaic (8, 9). Mosaicism might also underlie false-negative results in *APC* mutation screening because of the mutation detection level by usual techniques of screening (mainly sequencing). Mutations that appear de novo could represent germinal mosaicism in one of the parents. It is mandatory to test the offspring if there are affected individuals as the mutation is easier to detect. For this reason, testing a younger affected generation, if available, is preferable to testing the first affected generation, even if the polyposis phenotype is not yet completely expressed in the second generation, but a mutation carrier status is likely due to the presence of some symptoms as in our case (mandible osteomas). We determined that the proband had 2.5% of mutated cells in tested tissues (lymphocytes, normal colonic cells, and oral epithelium). Because the mutation was easily detectable with DNA extracted from colon adenomas as previously described in other case of *APC* mosaicism (9), frozen adenomas shall be conserved by the pathologist in all polyposis in order to perform DNA analysis if necessary.

Direct sequencing is not the best technique for mosaicism detection as it has a sensitivity of 10–20% for detecting a mutated allele (8). Pre-screening techniques seem to be more sensitive than direct sequencing in the mosaicism mutation detection (8). We confirmed that the HRM method was more efficient in this application (detection threshold at 2.5–5% for the mutated allele). Another study evaluated HRM sensitivity to 5% (24). The mutation analysis of genes with high proportion of de novo mutation should be performed by pre-screening technique like HRM.

We did not find any large rearrangement of *MUTYH* gene in cases with available RNA. In the literature, no case of large genomic rearrangement of *MUTYH* has been described yet (25, 26). The structure of the gene and the small introns length do not favour the occurrence of large rearrangement. The search for new candidate tumour suppressor genes by exploration of the Wnt pathway was not successful. No mutation was identified on *SFRP1*, *WIF1*, *PPP2R1B* and *AXIN2*. Sequencing of *AXIN2* found a rare variant c.1235A>G (p.Asnn412Ser) that we classed as polymorphism by studying a control healthy population. This variant has already been identified in a patient with unknown adenomatous polyposis and also classified as non-pathogenic by the authors on conservation species and physicochemical arguments (27). Moreover, the patient did not have dental abnormalities as they have been described associated to the polyposis due to this gene (27, 28). However, the exploration of 82 CRC and 27 polyposes, did not find any mutation on *AXIN2* gene (29,
30). The germline AXIN2 mutation is probably a rare event responsible of polyposis associated with severe tooth agenesis.

Screening of the TGF-β superfamily members, identified a pathogenetic mutation in BMPRIA on a previously identified patient with an adenomatous polyposis and an SMAD4 mutation on a patient with a first histological diagnosis of serrated polyposis. Mutations in the BMPRIA or SMAD4 gene are already known to predispose to juvenile polyposis. These results are consistent with second histological examination of the specimen of both cases. Some inflammatory or unclassified polyps are compatible with juvenile polyps. The histological classification of polyposis is important because it points genetic investigations. Referral for molecular genetic testing should be performed in a targeted manner after appropriate endoscopic and histological workup has been carried out. These results confirm that pathological examination is critical as already observed by Sweet et al. (31). If nosological doubt remains, a second look on the polyps from a skilled pathologist is recommended.

After these investigations, some polyposis still remains genetically unexplained. Other genes (of the Wnt signalling pathway or TGF-β/bone morphogenic protein (BMP) pathway) may be involved and several other candidates can be proposed. For example, MCC (mutated in colorectal cancer) had already been described in colorectal tumourigogenesis (32) or PTEN. Whole exome next generation sequencing could be performed in familial cases of unexplained polyposis. Mechanisms other than coding region mutation can be involved: germline epigenetic inactivation, miRNA, low frequency of susceptibility variants (33–36).

We have to report some bias in this work. The number of patients is relatively low even if such unexplained polyposis patients are very rare. We did not screen the MUTYH gene in the group of patients with an unexplained polyposis but mutations in this gene have not been found in a recent work on serrated polyposis (31).

A suspected diagnosis based on clinical and histological features is a requirement for rational, targeted testing for mutation. Misinterpretation of histological findings can misled genetic testing. Demonstration of a causal mutation is essential for the differential diagnosis, assessment of the risk of recurrence, monitoring, and genetic counselling (pre-symptomatic diagnosis). Close collaboration between human geneticists, pathologists and gastroenterologists is necessary for genetic predisposition to be identified.

References

21. Neklason DW, Solomon CH, Dalton AL, Kuwada SK, Burt RW. Intron 4 mutation in APC gene results in splice