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ORIGINAL ARTICLE

Filling the gap: A thorough investigation for the genetic diagnosis of unsolved polyposis patients with monoallelic *MUTYH* pathogenic variants

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Abstract

Backgrounds: *MUTYH*-associated polyposis (MAP) is an autosomal recessive disease caused by biallelic pathogenic variants (PV) of the *MUTYH* gene. The aim of this study was to investigate the genetic causes of unexplained polyposis patients with monoallelic *MUTYH* PV. The analysis focused on 26 patients with suspected MAP, belonging to 23 families. Ten probands carried also one or more additional *MUTYH* variants of unknown significance.

Methods: Based on variant type and on the collected clinical and molecular data, these variants were reinterpreted by applying the ACMG/AMP rules. Moreover, supplementary analyses were carried out to investigate the presence of other variants and copy number variations in the coding and promoter regions of *MUTYH*,

as well as other polyposis genes *(APC*, *NTHL1*, *POLE*, *POLD1*, *MSH3*, *RNF43*, and *MCM9*).

Results: We reclassified 4 out of 10 *MUTYH* variants as pathogenic or likely pathogenic, thus supporting the diagnosis of MAP in only four cases. Two other patients belonging to the same family showed a previously undetected deletion of the *APC* gene promoter. No PVs were found in the other investigated genes. However, 6 out of the 18 remaining families are still interesting MAP candidates, due to the co-presence of a class 3 *MUTYH* variant that could be reinterpreted in the next future.

Conclusion: Several efforts are necessary to fully elucidate the genetic etiology of suspected MAP patients, especially those with the most severe polyposis/tumor phenotype. Clinical data, tumor molecular profile, family history, and polyposis inheritance mode may guide variant interpretation and address supplementary studies.

KEYWORDS

monoallelic, *MUTYH*, pathogenic variant, polyposis

1 | INTRODUCTION

MUTYH-associated polyposis (MAP) is an autosomal recessive disease caused by biallelic pathogenic variants (PVs) of the *MUTYH* gene (OMIM #604933; Al-Tassan et al., 2002; Mazzei et al., 2013). Clinically, MAP resembles the familial adenomatous polyposis (FAP), caused by constitutional heterozygous PVs in the *APC* gene (OMIM #611731). The clinical phenotype is characterized by a wide variability in polyp burden, age of onset, and colorectal cancer (CRC) incidence. On average, MAP is relatively mild, in most cases mimicking AFAP, the attenuated form of FAP (Nielsen et al., 2012). Concerning histopathology, polyposis is characterized mainly by tubular or tubulovillous adenomas located throughout the colon, although hyperplastic polyps and sessile serrated adenomas are also a common finding in MAP. In the absence of timely surveillance, the lifetime CRC risk of MAP patients is greatly increased in comparison to the general population (80%–90%) and ~60% of MAP patients with polyposis have CRC at first presentation (Nielsen et al., 2011). Of note, almost one third of individuals with biallelic germline *MUTYH* PVs identified in population-based CRC studies develop CRC in the absence of polyposis (Nielsen et al., 2012; Venesio et al., 2012).

It has been suggested that monoallelic carriers of *MUTYH* PVs may have a slightly increased risk for CRC. However, study results are conflicting and the magnitude of this risk is still controversial. Indeed, available epidemiological data, derived from case–control studies and meta-analyses, rarely supported a significant increase of CRC risk in monoallelic individuals identified in the general population (Nielsen et al., 2011). Instead heterozygous carriers related to a MAP patient seem to have a small but higher risk, posing some uncertainty on whether specialized surveillance is warranted. However, data do not indicate significant association of a single PV of the *MUTYH* gene with a clinical picture of colorectal polyposis (Win et al., 2011).

The family history of MAP patients is also extremely variable, ranging from an apparent sporadic condition to several affected siblings and/or family members. This wide heterogeneity in terms of both disease and familial phenotype makes difficult to define specific and narrow eligibility criteria for genetic testing and the large majority of *MUTYH* gene tests produce negative/uninformative results (Terlouw et al., 2020).

To date, the total number of public variants reported in the LOVD-InSiGHT database (Out et al., 2010) are 3657, corresponding to 629 unique variants, spanning from clear-cut PVs to common polymorphisms.

Despite the high frequency of PVs in relevant hot spots, such as the founder p.(Tyr179Cys) and p.(Gly396Asp) missense variants (Aretz et al., 2014), the genetic diagnosis of MAP is not always straightforward (Ricci et al., 2017). Other rare or private point mutations are often detected, that are difficult to be correctly interpreted. According to the literature, in contrast to mismatch repair gene variants, no gene-specific interpretation criteria have been proposed and the available *MUTYH* databases are less curated. This adds further complexity in the process leading to genetic diagnosis.

From a practical point of view, demonstrating inactivation of a single allele is not sufficient to genetically confirm or exclude the diagnosis of MAP. The aim of the study is to investigate the genetic causes of unexplained polyposis cases with monoallelic *MUTYH* PV.

2 | MATERIALS AND METHODS

2.1 | Patients

The analysis focused on 26 patients with suspected MAP, belonging to 23 families, tested in our Laboratory and enrolled in a time frame of 19 years (2001–2019) from different centers. This series consisted of patients affected by colorectal polyposis of variable severity, with or without CRC, with positive or negative tumor family history. They were selected from our dataset, including about 500 tested patients, among which 91 were genetically proven (biallelic carriers) MAP patients. All 26 selected patients carried a single *MUTYH* PV, with no evidence of certain biallelic inactivation. As a starting point, we considered definitely pathogenic only the truncating variants and a few selected missense, splicing, and inframe deletions variants, in accordance with literature data and mutation databases (Out et al., 2010; Ricci et al., 2017).

2.2 | DNA Sequencing and MLPA

Patients were studied for germline alterations of the *MUTYH* gene on blood DNA. All variants were annotated to LRG_220 reference sequences: NM_001128425.1 and NG_008189.1. Screening for point mutations was carried out by Sanger sequencing and/or target resequencing based on next generation sequencing (NGS) techniques, depending on the time of the enrolment. NGS libraries were sequenced on a Miseq platform (Illumina, Inc.). Reads alignment to the reference genome (GRCh37/hg19) and variant calling were performed by using the MiSeq Reporter software (Illumina, Inc.); variants were annotated with VariantStudio 3.0 (Illumina, Inc.). We used two custom targeted panels covering all coding sequences and intron–exon boundaries of the major polyposis and CRC susceptibility genes: a TruSeq Custom Amplicon (TSCA; Illumina Inc.), an amplicon-based NGS assay targeting the coding region and UTR of nine genes (*APC*, *BMPR1A*, *MLH1*, *MSH2*, *MSH6*, *MUTYH*, *PTEN*, *SMAD4*, and *STK11)*; an IDT panel (Integrated DNA Technologies, Inc) for Illumina Nextera Flex for Enrichment based on probes targeting all exons and splicing sites of the same genes covered by the previous panel plus six additional genes (*CDH1*, *MSH3*, *NTHL1*, *PMS2*, *POLD1*, and *POLE*). Differently from TSCA, this hybrid capture-based NGS assay performs well in detecting copy number variants (CNVs) at the analyzed loci.

Multiplex Ligation-dependent Probe Amplification (MLPA) P378 MUTYH SALSA® Probemix (MRC-Holland®) analysis was also used to detect CNVs, such as large gene deletions/duplications. Data were analyzed with GeneMapper® (Applied Biosystem/Life Technologies) and Coffalyser. Net (MRC-Holland) softwares. In addition, *APC* and *GREM1* CNVs were investigated with the P043-E1 APC SALSA® MLPA Probemix (MRC-Holland®).

Standard bidirectional Sanger sequencing was used for testing the *MCM9* and *RNF43* polyposis genes (primers and PCR conditions on request).

2.3 | Annotation and variant classification

Variants were reported according to the nomenclature recommendations of the Human Genome Variation Society (https://varnomen.hgvs.org/; den Dunnen et al., 2016). Existing variant evidences were obtained by consulting two *MUTYH* gene mutation databases, that is, ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and LOVD-InSiGHT (https://www.insight-group.org/variants/databases/). All variants with an allele frequency <0.01 were further categorized using the five-tier system according to the guidelines of the ACMG/AMP (American College of Medical Genetics and Genomics and the Association for Molecular Pathology; Richards et al., 2015). The variants were classified into pathogenic (class 5), likely pathogenic (class 4), uncertain significance (class 3), likely benign (class 2), and benign/polymorphism (class 1).

2.4 | *MUTYH* and *APC* genes promoter analysis

MUTYH gene promoter area is very large and extends about 5 kb before the ATG (Köger et al., 2019; Out et al., 2010). The screening was carried out by the amplicon-based NGS and/or by PCR and standard Sanger sequencing. For amplification of the putative promoter region, we used a forward primer annealing to the upstream sequence (Prom_1F: 5'-CCTTGGGGTTGGGTATGG-3') and a reverse primer annealing to intron 1 (Prom_1R: 5'-TCGGTCGCTCTTACACCC-3'), giving an amplicon of 1809 bp. PCR was performed with GoTaq® Green Master Mix (Promega Corporation) with 40 cycles of amplification at the following conditions: 94°C 30″, 60°C 30″, 72°C 150″. Three forward internal primers (5'-GAGGGCTGGCACTAAAGAGA-3',

5 ' - C C T C C C C A G A T G T T G T G C T T - 3 ' , 5'-TGCAGGAAACATTTTGTAGGGCT-3') and one reverse internal primer (5'-GGAAGCCGCTCACCGTC-3') were also employed for sequencing. Data were analyzed with the SeqScape™ software v3.0 (Applied Biosystem).

The promoter 1B of the *APC* gene was similarly analyzed. For the PCR and sequencing, we used a forward primer annealing to promoter (*APC* 5′UTR-F: GCCAGTAAGTGCTGCAACTG) and a reverse primer annealing to intron 1 (*APC* 5′UTR-R: GGAGAGGGTGAGACATGGAG) giving an amplicon of 680 bp. PCR was performed with 40 cycles of amplification at the following conditions: 94°C 30″, 58°C 30″, 72°C 60″.

2.5 | Clinical and somatic data collection

Several clinical and molecular parameters were examined from the available clinical records: sex, age of diagnosis, number and histology of polyps, CRC, other tumors, extracolonic manifestations, family history, transmission mode, and molecular profile of tumors. Additional somatic analyses of target genes, such as *KRAS* and *APC* (Lipton et al., 2003), were carried out on DNA extracted from paraffin-embedded tumor tissues (Viel et al., 2017).

3 | RESULTS

3.1 | *MUTYH* patient genotype

Twenty-six patients with monoallelic PVs of the *MUTYH* gene were selected from our in house series. These PVs included 13 c.1187G>A p.(Gly396Asp), four c.933+3A>C p.(Gly264Trpfs*7), and two each of the c.536A>G p.(Tyr179Cys), c.734G>A p.(Arg245His), and c.1147delC p.(Ala385Profs*23) variants. The c.312C>A p.(Tyr104*), c.1012C>T p.(Gln338*), and c.1437_1439delGGA p.(Glu480del) variants were each present in only one carrier.

Sixteen patients (13 families) were just monoallelic heterozygous carriers of a *MUTYH* PV, while nine probands carried also a second *MUTYH* variant of unknown significance and one proband (FAP347) had three different additional variants (Table 1). By testing family members, at a later stage, position *in trans* could be deduced in three cases (Table 1). At the time of enrolment and genetic testing, all these cases were also reported as *APC* wild type.

3.2 | Clinical phenotype, family history, and tumor molecular profile

Evaluation of the clinical parameters reported in detail on Table 2 showed that the average age of polyposis onset was 50 years. Two patients had <5 polyps, 11 patients had a number of polyps between 5 and 30, eight patients between 30 and 100, four patients >100, whereas in one remaining case the number of polyps was not indicated. Most patients (18) had adenomas, five patients had mixed polyposis, with histologically confirmed serrated polyps in two of them. In three patients the histology was not known. Twelve patients had CRC in addition to polyps, one patient manifested adenomas in the duodenum and three patients also had cancers in other sites (duodenum, breast, and endometrium). A family history of polyposis or CRC was reported in two and six patients, respectively, whereas nine patients had a positive family history of both polyposis and CRC. However, the polyposis inheritance pattern suggested by the pedigrees was autosomal recessive or dominant in only four and six cases, respectively.

The medical reports of the two siblings of family AV158 described in both cases somatic molecular profiles characterized by microsatellite stability, presence of *BRAF* V600E and absence of *RAS* mutations. On the other hand, previous targeted NGS test on an adenomatous polyp of FAP789 showed *APC* and *KRAS* mutations derived from G>T transversion (Viel et al., 2017; Table S1).

3.3 | Revision of *MUTYH* variant interpretation

Based on variant type and on clinical and molecular phenotype, the *MUTYH* variants of unknown significance (i.e., those reported as Variant 2 in Table 1) were tentatively reinterpreted by applying the ACMG/AMP five-tier classification system. This approach resulted in the reclassification of two missense variants as pathogenic (class 5), one missense and one synonymous/splicing substitutions as likely pathogenic (class 4), one synonymous variant as likely benign (class 2); the other seven variants remained of uncertain significance (class 3; Table 3). Based on these results, the genetic diagnosis of MAP was confirmed only in the four patients carrying the variants reclassified as class 5 and 4, that is, c.544C>T p.(Arg182Cys), c.545G>A p.(Arg182His), c.721C>T p.(Arg241Trp), and c.690G>A p.[Val193_Gln230del; Gln230=] (Table 3; Figure 1).

TABLE 1 *MUTYH* gene variants in 26 suspected MAP patients

Family ID	Individual ID	Variant 1_cDNA	Variant 1_protein	Variant 2_cDNA	Variant 2_protein
AL01	FAP25	c.1187G>A	p.(Gly396Asp)		
AL01	FAP26	c.1187G>A	p.(Gly396Asp)		
PD30	FAP266	c.1012C>T	p.(Gln338*)		
AV59	FAP347	c.1187G>A	p.(Gly396Asp)	c.899G>T c.932G>A c.1431G>C	p.(Cys300Phe) p.(Arg311Lys) p.(Thr477=)[b]
VA12	FAP352	c.933+3A>C	p.(Gly264Trpfs*7)		
VA12	FAP470	c.933+3A>C	p.(Gly264Trpfs*7)		
MO02	FAP544	c.1187G>A	p.(Gly396Asp)		
MO05	FAP547	c.1187G>A	p.(Gly396Asp)		
AN19	FAP715	c.933+3A>C	p.(Gly264Trpfs*7)	c.287T>C	p.(Phe96Ser)
AV116	FAP789	c.1187G>A	p.(Gly396Asp)	c.544C>T	p.(Arg182Cys)[a,b]
TN31	FAP805	c.1187G>A	p.(Gly396Asp)		
FC01	FAP842	c.1147delC	p.(Ala385Profs*23)		
VR37	FAP844	c.1187G>A	p.(Gly396Asp)	c.545G>A	p.(Arg182His)[b]
VA35	FAP855	c.536A>G	p.(Tyr179Cys)	c.721C>T	p.(Arg241Trp)[b]
TN35	FAP865	c.1187G>A	p.(Gly396Asp)		
TO03	FAP882	c.1437_1439delGGA	p.(Glu480del)	c.248C>T	p.(Ser83Leu)[a]
AV142	FAP918	c.933+3A>C	p.(Gly264Trpfs*7)		
AN33	FAP926	c.1187G>A	p.(Gly396Asp)		
PR03	FAP1000	c.312C>A	p.(Tyr104*)	c.565G>A	p.(Gly189Arg)
AV157	FAP1049	c.1187G>A	p.(Gly396Asp)		
AV158	FAP1051	c.734G>A	p.(Arg245His)		
AV158	FAP1059	c.734G>A	p.(Arg245His)		
TN47	FAP1058	c.536A>G	p.(Tyr179Cys)	c.577-5A>G	p.?
AN63	FAP1081	c.1147delC	p.(Ala385Profs*23)	c.690G>A	p.[Val193_Gln230del, Gln230=][a,b]
CN12	FAP1129	c.1187G>A	p.(Gly396Asp)		
AV181	FAP1193	c.1187G>A	p.(Gly396Asp)	c.1640delC	p.(Ala547Glufs*24)

Note: MUTYH gene reference sequence: NM_001128425.1.

[a]Verified in trans by variant segregation analysis.

[b]Variants reclassified with ACMG/AMP criteria according to data reported in Table 3.

3.4 | Additional gene testing

Supplementary sequence and MLPA analyses were then carried out in order to fully explore *MUTYH* and other known polyposis genes, by using the best performing techniques currently available in our Laboratory. On the whole, all but two samples (FAP26 and FAP352) were sequenced with at least one NGS assay (Table S2). A summary of the investigated polyposis genes and methods are given in Table S3. *POLD1* and *POLE* analysis of nine samples was limited to Sanger sequencing of the hot spot regions in the exonuclease domains.

The search for mutations in other well established polyposis genes included in NGS panels yielded rare heterozygous variants of *NTHL1*, *POLE*, and *MSH3* (Table 4). No other relevant variants were observed in the coding sequence of the *APC* and *POLD1* tested genes. Moreover, CNV analyses, carried out by MLPA or targeted capture NGS, did not detect large deletions/duplications of *MUTYH* alleles, while using the APC Probemix version E1 we identified the *APC* variant g.(?_112071072)_(112071494_?) in first cousins FAP25 and FAP26 (Figure 2). This variant corresponds to Promoter 1B deletion on transcript NM_001127511.2, and was missed by analyses with the previous version of the *APC* probemix, not including this promoter region. *GREM1* duplication (Lieberman et al., 2017) was instead excluded in the 14 samples analyzed by the same MLPA Probemix.

TABLE 2 Clinicopathological characteristics and family history

Family ID	Individual ID	Sex	Proband/Relative	Inheritance	No of polyps	Type of polyps	Age at diagnosis	CRC site	Other tumors	Polyps and/or CRC family history[a]
AL01	FAP25	M	prob	AD	>100	Not indicated	29	Colon		x
AL01	FAP26	F	rel	AD	>100	Adenomas	19		Duodenum	x
PD30	FAP266	M	prob	no	>100	Mixed	75	Ascending colon		
AV59	FAP347	M	prob	no	30–100	Adenomas	46			
VA12	FAP352	F	prob	AD	5–30	Adenomas	45	Colon		x
VA12	FAP470	M	rel	AD	5–30	Adenomas	55			x
MO02	FAP544	M	prob	no	5–30	Adenomas	35			
MO05	FAP547	M	prob	no	5–30	Adenomas	62	Caecum	Duodenal adenomas	x
AN19	FAP715	M	prob	no	5–30	Mixed	48	Caecum		
AV116	FAP789	M	prob	no	30–100	Adenomas	53			x
TN31	FAP805	F	prob	no	5–30	Adenomas	65			
FC01	FAP842	F	prob	AR	Not indicated	Not indicated	58			x
VR37	FAP844	F	prob	no	30–100	Adenomas	50			x
VA35	FAP855	M	prob	no	>100	Adenomas	41	Colon	Breast	x
TN35	FAP865	F	prob	no	5–30	Adenomas	65			
TO03	FAP882	M	prob	no	30–100	Mixed	57			x
AV142	FAP918	M	prob	no	5–30	Adenomas	64			
AN33	FAP926	M	prob	AD	<5	Adenomas	43			x
PR03	FAP1000	M	prob	AR	30–100	Adenomas	59			x
AV157	FAP1049	M	prob	no	30–100	Adenomas	79	Descending colon		
AV158	FAP1051	F	prob	AR	5–30	Mixed (serrated)	38	Ascending colon		x
AV158	FAP1059	M	rel	AR	30–100	Mixed (serrated)	32			x
TN47	FAP1058	F	prob	no	30–100	Adenomas	35	Colon		x
AN63	FAP1081	F	prob	no	5–30	Adenomas	51	Colon	Endometrium	
CN12	FAP1129	F	prob	AD	<5	Not indicated	52	Colon		x
AV181	FAP1193	M	prob	no	5–30	Adenomas	41	Sigmoid colon		x

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; no, no evidence of AD or AR polyposis inheritance in the family.

[a]Presence of at least one first- or second degree relative with polyps or CRC.

TABLE 3 *MUTYH* variant classification

ID	cDNA	Protein	ACMG/AMP	Class
FAP347	c.899G>T	p.(Cys300Phe)	PM2 PP3	3
FAP347	c.932G>A	p.(Arg311Lys)	PM2 BP4	3
FAP347	c.1431G>A	p.(Thr477=)	BS1 BP4 BP6 BP7	2
FAP715	c.287T>C	p.(Phe96Ser)	PM2 PP3	3
FAP789	c.544C>T	p.(Arg182Cys)	PS3 PM2 PM3 PP3 PP4	5
FAP844	c.545G>A	p.(Arg182His)	PS3 PM2 PM3 PP3	4
FAP855	c.721C>T	p.(Arg241Trp)	PS3 PM2 PM3 PP3 PP5	5
FAP882	c.248C>T	p.(Ser83Leu)	PM2 PM3 BP4	3
FAP1000	c.565G>A	p.(Gly189Arg)	PM2 PP3	3
FAP1058	c.577-5A>G	p.?	PM2 PP3	3
FAP1081	c.690G>A	p.(Gln230=); p.(Val193_Gln230del)	PM2 PM3 PP3 PP5	4
FAP1193	c.1640delC	p.(Ala547Glufs*24)	PM2 PM4	3

Note: MUTYH gene reference sequence: NM_001128425.1

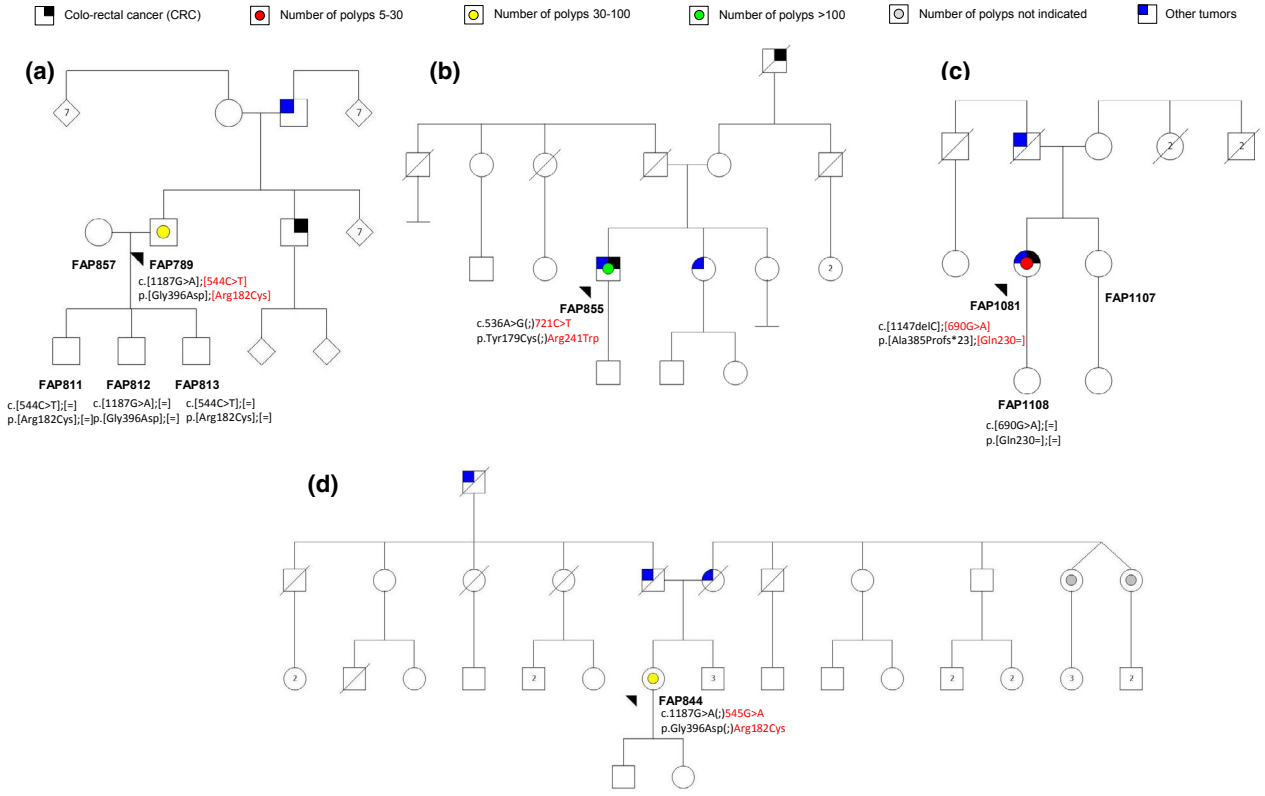

FIGURE 1 Pedigrees of the four MAP families: (a) AV116, (b) VA35, (c) AN63, (d) VR37. *MUTYH* genotypes are indicated below the symbols of the tested patients. The reclassified PVs are indicated in red. "Other tumors" means tumors other than CRC; see Table 2 for patient details

Additionally, *MUTYH* promoter sequencing analysis on 21 patients detected only the c.-1024G>A p.? variant in FAP347 (Table 4). Finally, Sanger sequencing of the 5'UTR region of *APC* gene on 15 patients, lacking this data from NGS analyses, confirmed absence of rare significant variants in the promoter of this gene.

According to the ACMG/AMP criteria, only the *APC* promoter 1B deletion identified in two members of the same family was pathogenic and causative of the polyposis phenotype (Table 4).

The search for mutations in other suspected polyposis genes was then restricted to a single selected family with a peculiar clinical phenotype. *RNF43* (McCarthy et al.,

TABLE 4 Summary of the gene variants identified by additional analyses and interpreted with the ACMG/AMP criteria

Individual ID	GENE	DNA	Protein	Class
FAP25	*APC*	NG_008481.4 g.(?_112071072)_(112071494_?)[a]	p.?	5
FAP26	*APC*	NG_008481.4 g.(?_112071072)_(112071494_?)[a]	p.?	5
FAP347	*MUTYH*	NM_001128425.1 c.−1024G>A	p.?	3
FAP347	*NTHL1*	NM_002528.7 c.274C>T	p.(Arg92Cys)	3
FAP715	*MSH3*	NM_002439.5 c.1896A>G	p.(Lys632=)	2
FAP1058	*MSH3*	NM_002439.5 c.190C>G	p.(Pro64Ala)	3
FAP1058	*POLE*	NM_006231.3 c.4477G>A	p.(Ala1493Thr)	3
FAP1058	*POLE*	NM_006231.3 2026+9C>T	p.(=)	2

[a]*APC* deletion was defined by two MLPA probes mapping on Promoter 1B.

2019) sequencing was carried out on FAP1051 and her brother FAP1059, because of the presence of serrated polyps; in addition, they were also tested for *MCM9* variants (Goldberg et al., 2015), because the female also presented hypergonadotropic hypogonadism. These two patients resulted wild type for both genes.

4 | DISCUSSION

In this article, we report the case of 26 patients with suspected MAP that were initially tested in a clinical setting for *MUTYH* variants, without confirmation of genetic diagnosis, due to the presence of only one PV, along with a second dubious variant in 10 patients. A genetic report was produced, including results and clinical interpretation.

For this study, we have revised the variants in the light of updated knowledge and using a standardized classification method. Clinical and genetic data allowed us to confirm diagnosis of MAP in only 4/26 patients. Therefore the remaining 22 cases are still NON-MAP patients, according to current criteria. The ACMG/AMP rules were applied to update the interpretation of the class 3 variants. These criteria were sufficient to support class 5 or class 4 for the second *MUTYH* variant of probands FAP789, FAP844, FAP855, and FAP1081. Functional impairment of MUTYH protein is a strong evidence of pathogenicity but, to date, functional data are available for only a minority of variants. In this study, three missense variants could be classified as pathogenic or likely pathogenic by incorporating the ACMG/AMP PS3 evidence derived from functional invitro analyses (Komine et al., 2015). The four confirmed MAP patients did not show obvious autosomal recessive inheritance of polyposis, but they all had a rather severe clinical phenotype, characterized by significant polyp burden, and/or CRC and consistent with the diagnosis of MAP. Moreover, the spectrum of somatic mutations of FAP789 tumor was marked by the typical G>T transversions of *APC* and *KRAS* genes that are consequent to MUTYH functional loss (Viel et al., 2017). The added value of tumor NGS analysis is unquestionable, because the molecular tumor profile and the mutational signature could aid in judging the possible pathogenicity of the underlying constitutional variants. Unfortunately, this is still not the standard of care in the diagnostic setting. The somatic tumor analysis could also be relevant to detect *APC* somatic mosaicism, as previously demonstrated (Jansen et al., 2017). However, this possibility was not explored due to the lack of suitable tissues, that is, multiple adenomas, carcinomas and normal intestinal mucosa.

We are aware that demonstrating the *in trans* phase of the two *MUTYH* PVs is mandatory for a definitive genetic diagnosis of MAP. At present, this is confirmed only for patients FAP789 and FAP1081, but we hope to proceed soon with genetic testing in relatives for the other two patients (FAP844 and FAP855), to ensure that also these index cases are truly biallelic carriers. However, databases and/or literature data have already reported detection of the c.545G>A and c.721C>T variants in compound heterozygosity with other known PVs (Guarinos et al., 2014; Jones et al., 2009).

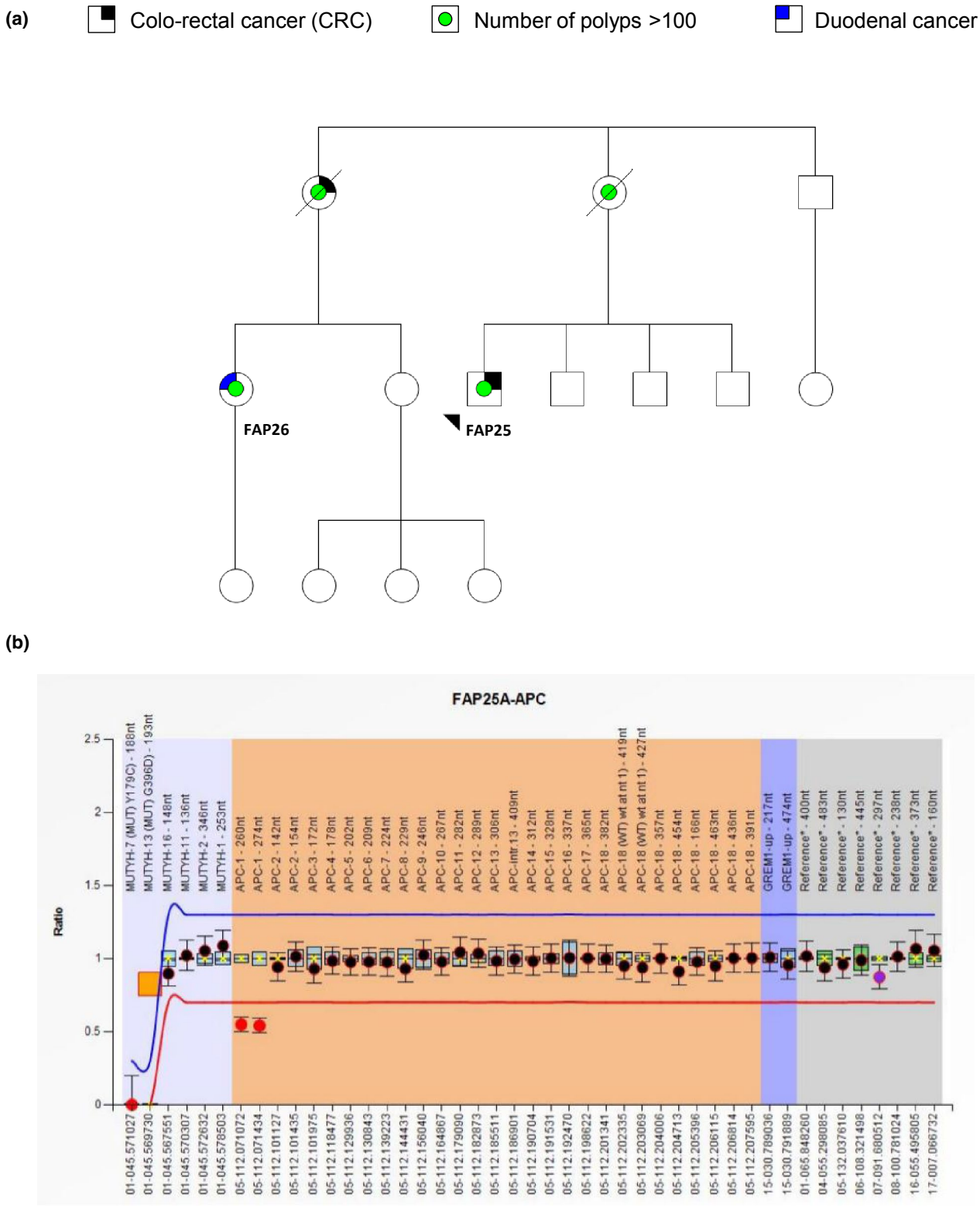

FIGURE 2 (a) Pedigree of the FAP AL01 family; (b) MLPA assay showing deletion of promoter 1B of the *APC* gene

The silent variant of patient FAP347 was downgraded to likely benign (class 2), class 3 was instead confirmed for the remaining seven variants in six patients. This last group included also the frameshift mutation of patient FAP1193 (c.1640delC), because it is located at the extreme 3' end of the gene and is predicted to cause protein extension by 20 more amino acids on the new reading frame, with uncertain impact on its function.

Reinterpretion/reclassification of the gene variants is an important challenge in medical genetics. It should be

responsibility of the diagnostic laboratories, but there is still no formal policy regarding this issue (El Mecky et al., 2019).

The methods used for the initial search of the *MUTYH* gene variants was dependent on the time of the patient enrolment, but then it was possible to repeat or expand sequence and CNV analyses in selected cases with available DNAs, in order to increase test sensitivity and to explore other predisposing genes. Whenever possible, additional analyses carried out on this series of cases aimed at investigating (a) DNA coding sequence variants and CNVs of the *MUTYH*, *APC*, and other known polyposis genes; (b) promoter variants of the *MUTYH* and *APC* genes; and (c) pathogenic significance of the identified variants.

On the whole, 24 out of 26 patients were tested by amplicon-based and/or hybrid capture-based NGS assays. All patients were tested for *MUTYH* and *APC* with several methods. Interestingly, the NGS coverage analysis showed a decreased read depth in exon 1 of *APC* in FAP25, suggesting a large deletion. Therefore, FAP25 and his cousin FAP26 were tested again with MLPA, which confirmed the suspected deletion of Promoter 1B in both DNA samples. The presence of this PV in *APC* is added to the single PV of *MUTYH*, found several years earlier, and completely changes the terms of the matter, allowing the diagnosis of FAP instead of MAP. It should be noted that one CRC and one duodenal cancer were diagnosed in this family and, according to literature, no gastric involvement has been reported (Lin et al., 2015; Marabelli et al., 2017). The used hybrid capture-based NGS panel had the advantage to detect also CNV variants and to cover additional polyposis genes, but did not include the entire UTR regions. For this reason, a wider tract of the *APC* promoter (Li et al., 2016) was further sequenced by the Sanger method in the majority of DNA samples, but the analysis did not detect significant variants.

Little is known about the role of *MUTYH* variants in the promoter region and, at present, there are no acknowledged PVs conferring disease risk, although a few variants able to compromise gene expression have recently been described (Köger et al., 2019). For this reason, the *MUTYH* gene promoter was similarly sequenced in order to assess the possible presence of additional *MUTYH* rare variants in this noncoding region. The aim was to further extend upstream the region analyzed by NGS beyond the positions covered with the hybrid capture panel (c.-127) or the amplicon panel (c.-370), but we only detected an additional rare variant of unknown meaning (c.-1024G>A p.?) in FAP347.

For completeness of data, sequencing of *NTHL1* (Weren et al., 2015), *MSH3* (Adam et al., 2016), *POLD1*, and *POLE* (Palles et al., 2013) genes was also executed by NGS and/or Sanger method on 25 samples, excluding FAP352 for biological material depletion. The tests showed heterozygous missense variants of uncertain significance of *NTHL1* gene in FAP347 and of *MSH3* and *POLE* genes in FAP1058. Silent, likely benign *MSH3* and *POLE* variants were also identified in FAP715 and FAP1058, respectively. Since both *NTHL1* and *MSH3* are recessive genes that are known to cause polyposis only in biallelic carriers, we believe rather unlikely a role of the detected heterozygous variants in these patients, unless a synergistic effect among different DNA repair genes is hypothesized. A digenic inheritance by mutations in the base excision repair genes *MUTYH* and *OGG1* was suggested years ago for hereditary CRC (Morak et al., 2011), but no additional convincing evidences on these two genes have been provided since then. The recent demonstration of coinheritance of monoallelic variants in *MSH6* and *MUTYH*, consistent with cosegregation with CRC, raises one more time the digenic inheritance as a cause of CRC genetic predisposition (Schubert et al., 2020). However, no appealing *MSH6* variants have been detected in our series of patients (data not shown).

The case of FAP347 is peculiar. In addition to the monoallelic PV of *MUTYH*, this patient showed a *NTHL1* variant and four additional rare variants in *MUTYH*, three in the coding region, and one in the promoter; nevertheless, we have not been able to classify his disease as MAP by applying the ACMG/AMP criteria. c.899G>T p.(Cys300Phe) is located on the FeS cluster loop domain, it has never been reported in any public database and bioinformatics prediction is particularly suggestive of pathogenicity, but no other data are available to allow upgrading of this variant to class 4. From the sequence of NGS reads we know that in this patient the c.899G>T p.(Cys300Phe) and c.932G>A p.(Arg311Lys) *MUTYH* variants are located *in cis* on the same allele, but we could not assess if they are *in trans* in respect to c.1187G>A p.(Gly396Asp).

The case of the siblings FAP1051 and FAP1059, both monoallelic *MUTYH* carriers with atypical clinical phenotypes (adenomatous and serrated polyps and hypergonadodropic hypogonadism in the female), is also particularly intriguing. The somatic profiles of the FAP1051 CRC and FAP1059 adenoma, lacking the typical *KRAS* G>T transversion, are against the hypothesis of an underlying MUTYH functional defect. On the other hand, during the family follow-up another young sister, who was *MUTYH* homozygous wild type, developed an early onset CRC in the absence of colorectal polyps. This suggests that other unknown predisposing genes are probably involved in this family. A role of the *RNF43* gene, rarely associated with the serrated polyposis (McCarthy et al., 2019), has been excluded. Similarly, we also excluded the hypothesis of *MCM9*, previously reported as a candidate risk gene of

hereditary mixed polyposis and CRC associated with primary ovarian failure (Goldberg et al., 2015).

In summary, of the 23 families (26 patients) selected for this study, four were confirmed MAP and one was shifted to FAP. These findings may have a relevant impact on the future clinical managements of the patients and their relatives. Of the 18 remaining families, six are still interesting MAP candidates due to the co-presence of a class 3 *MUTYH* variant that could be reinterpreted in the next future.

Our experience shows that the progresses in scientific knowledge and molecular analysis techniques offer diagnostic opportunities by constantly providing new and best performing methods with improved sensitivity. The case of family AL01 is emblematic, since it tested *APC* negative 15 years ago, but is now diagnosed as FAP, thanks to the new genotype–phenotype data published on the *APC* gene promoter (Lin et al., 2015; Marabelli et al., 2017) and the availability of diagnostic assays with superior detection capability.

5 | CONCLUSIONS

Several efforts are necessary to fully elucidate the genetic etiology of suspected MAP patients, especially those with the most severe polyposis/tumor phenotype. The possibility of a second *MUTYH* alteration, missed by the current gene testing methods, cannot be excluded in some patients. The use of wider gene panels including additional risk genes is warranted in some cases. Patient clinical phenotype, tumor molecular profile, family history, and polyposis inheritance mode may guide variant interpretation and address supplementary studies.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Anastasia Dell'Elice collected data, analyzed and interpreted the results, summarized the findings, prepared the figures and tables, and wrote the original draft. Giulia Cini contributed to the genetic investigation, literature review, data presentation, and manuscript writing. Mara Fornasarig, Franco Armelao, Isabella Mammi, and Italo Sorrentini recruited patients and collected clinical data. Daniela Barana, Francesca Bianchi, Guido Claudio Casalis Cavalchini, Antonella Maffè, Antonio Percesepe, and Monica Pedroni contributed to patient enrolment, data collection, and data analyses. Maria Grazia Tibiletti provided intellectual input and contributed to the interpretation of genetic findings. Roberta Maestro critically revised the manuscript. Michele Quaia contributed to genetic testing of patients and relatives. Alessandra Viel designed the study concept, coordinated and supervised the work, revised the manuscript, and acquired funding.

ETHICAL COMPLIANCE

The genetic testing protocol and use of DNA samples for research purposes was approved by the Local Independent Ethical Committee (CRO-15-1997). Written informed consent was obtained from all individuals enrolled in the present study.

DATA AVAILABILITY STATEMENT

The datasets supporting the conclusions of this article are included within the article and its additional file.

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