New insights into POLE and POLD1 germline mutations in familial colorectal cancer and polyposis

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Germline mutations in DNA polymerase ε (POLE) and δ (POLD1) have been recently identified in families with multiple colorectal adenomas and colorectal cancer (CRC). All reported cases carried POLE c.1270C>G (p.Leu424Val) or POLD1 c.1433G>A (p.Ser478Asn) mutations. Due to the scarcity of cases reported so far, an accurate clinical phenotype has not been defined. We aimed to assess the prevalence of these recurrent mutations in unexplained familial and early-onset CRC and polyposis, and to add additional information to define the clinical characteristics of mutated cases. A total of 858 familial/early onset CRC and polyposis patients were studied: 581 familial and early-onset CRC cases without mismatch repair (MMR) deficiency, 86 cases with MMR deficiency and 191 polyposis cases. Mutation screening was performed by KASPar genotyping assays and/or Sanger sequencing of the involved exons. POLE p.L424V was identified in a 28-year-old polyposis and CRC patient, as a de novo mutation. None of the 858 cases studied carried POLD1 p.S478N. A new mutation, POLD1 c.1421T>C (p.Leu474Pro), was identified in a mismatch repair proficient Amsterdam II family. Its pathogenicity was supported by cosegregation in the family, in silico predictions, and previously published yeast assays. POLE and POLD1 mutations explain a fraction of familial CRC and polyposis. Sequencing the proofreading domains of POLE and POLD1 should be considered in routine genetic diagnostics. Until additional evidence is gathered, POLE and POLD1 genetic testing should not be restricted to polyposis cases, and the presence of de novo mutations, considered.

INTRODUCTION

Estimates indicate that familial colorectal cancer (CRC) defined by the presence of two or more first-degree relatives affected with CRC involves over 20% of all cases (1–3). Nevertheless, CRC syndromes caused by known high-penetrance CRC genes collectively account for only 2–6% of all CRC cases. Germline mutations and epimutations in the DNA mismatch repair (MMR) genes MLH1, MSH2, MSH6 and PMS2 cause Lynch syndrome, explaining a proportion of hereditary non-polyposis CRC cases;
mutations in *APC* and *MUTYH* primarily predispose to multiple colonic adenomas, a benign precursor of CRC; a 40 kb upstream duplication in *GREM1* cause hereditary mixed polyposis; and several types of hamartomatous polyposes are explained by mutations in *SMAD4*, *BMPRIA*, *STK11* and *PTEN* (4–9). Nevertheless, there are still a number of CRC families suggestive of carrying a mutation in a high-penetration predisposition gene, but without mutations in the known genes. Among these, a number of familial adenomatous polyposis cases are not explained by germline mutations in *APC* or *MUTYH*.

Recently, using a combination of whole-exome sequencing and linkage analysis in probands with >10 adenomas by age 60 but no germline mutations in *APC*, *MUTYH* or the MMR genes, Palles et al. identified DNA polymerase ε (*POLE*; MIM #174762) and δ (*POLD1*; MIM #174761) mutations in individuals/families with multiple colorectal adenomas and CRC (10). In all, two pathogenic variants, *POLE* c.1270C>G (p.Leu424Val) (NM_006231) and *POLD1* c.1433G>A (p.Ser478Asn) (NM_002699), and an additional variant whose pathogenicity has not yet been determined, *POLD1* c.981C>G (p.Pro327Leu), were identified. All three genetic changes affect the proofreading (exonuclease) domain of the respective polymerase, suggesting deficient proofreading repair during DNA replication (10–13). After a comprehensive screening of the identified pathogenic mutations in over 3800 CRC patients of European ancestry enriched for a familial CRC history, multiple adenomas and early-onset disease, a total of 13 families with *POLE* or *POLD1* mutations in individuals/families with multiple colorectal adenomas and CRC (10). In all, two pathogenic variants, *POLE* c.1270C>G (p.Leu424Val) (NM_006231) and *POLD1* c.1433G>A (p.Ser478Asn) (NM_002699), and an additional variant whose pathogenicity has not yet been determined, *POLD1* c.981C>G (p.Pro327Leu), were identified. All three genetic changes affect the proofreading (exonuclease) domain of the respective polymerase, suggesting deficient proofreading repair during DNA replication (10–13).

In the study, we aimed to assess the prevalence of *POLE* p.L424V and *POLD1* p.S478N in polyposis and non-polyposis familial and early-onset CRC cases, and to add additional information to help define the phenotypic/clinical characteristics of mutated cases.

**RESULTS**

Neither *POLE* p.L424V nor *POLD1* p.S478N was identified in genetically uncharacterized familial non-polyposis CRC cases, including 581 MMR-proficient and 86 MMR-deficient cases. Likewise, *POLD1* p.S478N was not detected in 191 polyposis cases.

*POLE* p.L424V was identified in a polyposis family (Series no. 1) (Fig. 1A). The index case was a female patient diagnosed with CRC (pT2pN0pM0) and >35 colonic polyps at age 28. From a total of 33 polyps analyzed, 31 were adenomas, 1 a hyperplastic polyp and 1 a mixed polyp. At 30 years old, 2 years after the surgery, she had developed 8 additional adenomas. No genetic alterations in *APC* and absence of the common *MUTYH* variants had been identified. No loss of heterozygosity (LOH) of the *POLE* chromosomal region, analyzed with two informative microsatellites 1.13 Mb apart, and studying the allelic abundance of the mutated and wild-type alleles by SnPshot, was detected in tumor DNA extracted from the colon tumor developed by the mutation carrier (Supplementary Material, Fig. S1).

Based on the clinical findings of the proband, her father was subjected to a colonoscopy at age 56, which revealed a pT2pN0pM0 tumor (adenocarcinoma arising from a tubulovillous adenoma) at the proximal colon and one hyperplastic polyp. Her mother was diagnosed with breast cancer at age 36 and died at 41. No information on gastrointestinal clinical findings in the mother had been reported, and no colon cancer surveillance measures were followed in the maternal family branch. The study of cosegregation revealed that the father did not carry the p.L424V mutation, suggesting a different etiology for his CRC. Paternity was confirmed by microsatellite analysis (data not shown). Likewise, p.L424V was not identified in the DNA extracted from an archived cytology sample obtained from an affected node (metastasis) of the mother’s breast cancer. Therefore, these findings indicated that *POLE* p.L424V occurred as a de novo germline mutation in the index case.

On account of the mutation-screening method used in the Series no.2, consisting of sequencing exons 13 and 11 of *POLE* and *POLD1*, respectively, a novel genetic change, *POLD1* c.1421T>C (p.Leu474Pro), was detected in an Amsterdam II MMR-proficient family. The index case was a female patient diagnosed with a well-differentiated left colon cancer (pT2pN0pM0) and a synchronous gastrointestinal stromal tumor (GIST) in the large bowel at age 36. No polyps were ever found during surgical removal or follow-up. Her mother was diagnosed with endometrial cancer at age 52. A maternal aunt was diagnosed with metachronous CRC (pT3pN0pM0) and endometrial cancer (Stage IB) at ages 33 and 56, respectively, and no polyps were found in the intestinal tract during surgical intervention and follow-up. A maternal uncle was diagnosed with a gastric cancer at age 72, and his daughter died of a brain tumor at age 42. The maternal grandmother died from a bladder cancer at age 51 (Fig. 1B). Cosegregation analysis performed in the maternal aunt, diagnosed with CRC (33 years) and endometrial cancer (56 years), confirmed her status of heterozygous carrier. Therefore, the mother of the index case was an obligate mutation carrier.

The variant *POLD1* p.L474P is localized in a highly conserved residue located within the proofreading domain of DNA polymerase δ. *In silico* analysis using SnPs3D, PolyPhen-2, Condel and SIFT algorithms predicted relevant functional effects with scores of −3.36 (deleterious), 1 (probably damaging), 1 (deleterious) and 0 (damaging), respectively. Human *POLD1* p.L474 is the homologous residue of p.L479 in *Saccharomyces cerevisiae*. The mutation p.L479S Pol3 in this organism has been shown to cause a mutator phenotype (14). Moreover, human *POLD1* p.L474 is the paralogous residue of the human *POLE* p.L424, the residue where the recurrent *POLE* p.L424V mutation occurs (10). In summary, evidence from cosegregation, *in silico* predictions of the variant’s functionality and yeast functional assays strongly suggests a pathogenic nature for *POLD1* p.L474P.

Mutation screening of the driver genes *KRAS* (codons 12 and 13, and exons 3 and 4), *NRAS* (exons 2–4), and *BRAF* p.V600E in the colorectal tumor developed by the index case and in the endometrial tumors developed by her maternal aunt revealed no somatic mutations.
DISCUSSION

**POLE** p.L424V and **POLD1** p.S478N mutation screening in 858 Caucasian (Spanish) patients with CRC and/or colonic polyposis, enriched for a family history of colorectal tumors, multiple colonic polyps and/or early-onset disease, identified one carrier of **POLE** p.L424V. This accounts for 0.12% (1/858) of the total, 0.52% (1/191) of the polyposis cases, and 0.86% (1/116) of the adenomatous polyposes studied. Despite its infrequency and based on the simplicity of the test, our findings provide further evidence to advice that at least **POLE** p.L424V, as a recurrent mutation, should be tested in adenomatous polyposis cases without mutations in **APC** and **MUTYH**.

Together with the family identified in our series, a total of 14 families carrying the **POLE** p.L424V mutation have been reported and described in the literature (10). Eleven of them were CRC-only and/or polyposis families. Of the other two families previously described, an astrocytoma and tumors of the ureter, ovary and breast were reported in mutation carriers, or probable mutation carriers, who had also been diagnosed with at least two additional colorectal tumors (10). Recently, an additional carrier of a **POLE** mutation, the deletion c.5621_5622delGT, has been identified in a patient diagnosed with CRC at 26 years of age, with no further information reported about family history of cancer or polyposis (15). In our family, the p.L424V mutation occurred *de novo* and caused early-onset CRC (28 years) and adenomatous polyposis. To date, this is the first *de novo* case reported for **POLE/POLD1** germline mutations. Nevertheless, as occurs in 20% APC mutation carriers...
with an apparent de novo mutation (16), it could also be a consequence of a somatic mosaicism in one parent. Being POLE p.L424V a recurrently found mutation, it can be hypothesized that its recurrent nature may be the result of a founder effect. Even though this could still be true for certain instances, the existence of de novo p.L424V mutations supports the idea of a mutation hotspot, which may also originate non-founder recurrent mutated cases.

In this study, we also report a novel mutation, POLD1 p.L474P, identified in an Amsterdam II family without defects in the MMR system. This finding suggests that polymerase proofreading mutations in POLD1 explain a proportion of the uncharacterized hereditary non-polyposis CRC cases. This finding supports the screening of the genes, at least of POLD1, in non-polyposis CRC cases. In this regard, the term ‘polymerase proofreading-associated polyposis’ may be misleading and should be carefully used, at least until more POLE/POLD1 families are described and the full phenotypic spectrum of this syndrome is refined.

In addition to the family herein identified, carrying POLD1 p.L474P, three additional families with germline POLD1 mutations have been previously described, all of them carrier of p.S478N (10). In three of the four POLD1 families reported, including the one described here, two or more endometrial tumors have been diagnosed, indicating the importance of cancer surveillance of this type of tumor in POLD1 mutation carriers (10,17).

Tumors developed in the context of polymerase proofreading mutations, both germline and somatic, show an ultramutated, apparently microsatellite-stable phenotype, sometimes leading to over a million base substitutions per tumor. In these tumors, the mutation spectrum is changed, with a particular increase in the proportion of G : C to T : A and A : T to C : G transversions. Therefore, it would be expected to find numerous mutations in common CRC genes. However, except for rare mutations in APC, the frequency of mutations in other driver genes is low (13). Here, the screening of common KRAS, NRAS and BRAF mutations in two tumors (1 colorectal and 1 endometrial cancer) from two POLD1 p.L474P carriers, which also showed MMR proficiency, revealed no mutations, even when some of the most common mutations found in those genes in colorectal cancer, such as KRAS c.34G>T (p.G12C) or BRAF c.1799T>A (p.V600E), are transversions. This agrees with the results obtained by Palles et al. (10), where no mutations in driver genes were identified in 4 of 6 tumors from five POLD1 mutation carriers, and in 5 of 10 tumors from three POLE mutation carriers. Additional studies analyzing the mutation burden and the presence of mutations in additional known driver genes in the tumors developed by POLE and POLD1 mutation carriers, both CRC and endometrial, will provide a clearer picture of the somatic molecular landscape of this syndrome.

Our results, together with the information gathered so far, support the recommendation of sequencing the exons encoding the proofreading domains of POLE and POLD1 in all familial CRC and polyposis cases without mutations in the known predisposing genes. Based on our findings, POLE and POLD1 mutation screening should not be restricted to polyposis cases, and the presence of de novo mutations should be taken into account. Also, whenever endometrial cancer cases are reported in the family, genetic testing of POLD1 should be prioritized.

**MATERIALS AND METHODS**

**Patients**

A total of 858 familial/early-onset CRC and polyposis patients from 840 families were included in the analysis. Written informed consent was obtained from all subjects and the study received the approval of the Ethics Committees of the involved institutions.

**Series no.1**

A total of 612 familial and/or early-onset colorectal cancer and/or polyposis patients from 594 families without identified mutations in the known CRC or polyposis genes were included in the study. All had been referred to the Genetic Counseling Units of the Catalan Institute of Oncology in the Spanish region of Catalonia between 1999 and 2012. Referral was based on familial history of colorectal cancer or polyps, presence of early-onset colorectal cancer and/or personal history of polyposis at early age.

All non-polyposis cases (n = 524) had been previously tested for MMR deficiency, either by immunohistochemistry of the MMR proteins MLH1, MSH2, MSH6 and PMS2, and/or by PCR-based microsatellite instability analysis. MMR-deficient tumors (n = 86) did not present somatic MLH1 promoter methylation and/or the BRAF V600E mutation, thus suggesting a hereditary component. Nevertheless, no germline mutations were identified in the candidate MMR genes, according to the MMR protein expression pattern in the tumor. Clinical features of non-polyposis cases are shown in Table 1.

All polyposis patients (n = 88) had undergone MUTYH genetic testing of the three most recurrent genetic variants in Spanish population, i.e. p.Tyr179Cys, p.Gly396Asp and p.Glu410Glyfs*43 (NM 001128425.1) (18). If one of these three was detected, all the coding regions of MUTYH were subsequently sequenced. When the number of adenomas was >20, the APC gene, including exons and flanking regions, was also sequenced. Clinical features of polyposis cases are shown in Table 2.

**Series no.2**

Series no.2 consisted of 246 uncharacterized hereditary CRC and/or polyposis index patients. The 143 non-polyposis CRC patients included in the study were recruited through the Cancer Genetic Counseling Units of the Spanish region of Valencia between 2005 and 2013. Of them, 63 cases fulfilled the Amsterdam criteria (I or II) and were MMR-proficient. The remaining 80 cases were diagnosed with MMR-proficient non-polyposis CRC and had two or more first or second-degree relatives diagnosed with a Lynch syndrome-related tumor, regardless of age (Bethesda criterion no. 5) (Table 1). The status of MMR deficiency was assessed either by immunohistochemistry of the MMR proteins and/or by PCR-based microsatellite instability analysis. Biological samples and clinicopathological information were obtained from the Valencian Biobank Network and from the Hereditary Cancer Program of the Valencia Region, both in Spain.

A total of 103 polyposis cases were recruited through the EPIPOLIP project, which comprises a multicentric Spanish series (19). All cases were diagnosed with attenuated polyposis with >10 polyps and at least one first-degree relative affected with
MRR, mismatch repair; N, number of individuals; fam., number of families; Ams., Amsterdam criteria (I or II); Beth., Bethesda criteria; n.a., not available data; SD, standard deviation.

1Non-polyposis cases whose tumors showed microsatellite stability and intact expression of the MMR proteins MLH1, MSH2, MSH6 and PMS2.

2Non-polyposis cases whose tumors showed microsatellite instability and/or loss of expression of at least one MMR protein.

3Cases referred from the Department of Pathology (CSUB, IDIBELL) to the Hereditary Cancer Program (ICO, IDIBELL) based on tumor histopathological features suggestive of MMR deficiency, which was subsequently confirmed. Somatic promoter MLH1 methylation was discarded and/or the presence of BRAF V600E confirmed. No information on familial cancer history was available.

Table 2. Characteristics of the polyposis cases analyzed

<table>
<thead>
<tr>
<th>Criteria n (%)</th>
<th>N (fam.)</th>
<th>Ams. I</th>
<th>Ams. II</th>
<th>Beth.</th>
<th>n.a.</th>
<th>Age at cancer diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (± SD)</td>
<td></td>
<td></td>
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<tr>
<td>MMR-proficient</td>
<td>438 (423)</td>
<td>31 (7.1%)</td>
<td>11 (2.5%)</td>
<td>390 (89.0%)</td>
<td>6 (1.4%)</td>
<td>49.0 (± 12.6)</td>
</tr>
<tr>
<td>MMR-deficient</td>
<td>86 (86)</td>
<td>1 (1.2%)</td>
<td>4 (4.7%)</td>
<td>63 (73.3%)</td>
<td>18 (20.9%)</td>
<td>51.4 (± 13.3)</td>
</tr>
<tr>
<td>Total</td>
<td>143 (143)</td>
<td>17 (11.9%)</td>
<td>46 (32.2%)</td>
<td>80 (55.9%)</td>
<td>0</td>
<td>49.4 (± 11.6)</td>
</tr>
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</table>

CRC. All cases had previously undergone genetic testing of the three most frequent MUTYH genetic variants in Spanish population, by sequencing exons 7 and 13 of the gene. If one of these three was detected, all the coding regions of MUTYH were subsequently sequenced. The APC gene was analyzed by Sanger sequencing in all individuals with >10 adenomas.

**POLE p.L424V and POLD1 p.S478N screening**

In Series no.1, KASPar assays (KASP-By-Design genotyping assays, LGC group, Teddington, UK) were used to genotype the two mutations. Reactions were carried out in the LightCycler 480 real-time PCR detection system (Roche Diagnostics GmbH, Germany), including a corresponding positive control in each run. Positive controls for POLE p.L424V and POLD1 p.S478N were kindly provided by Professor Ian Tomlinson (The Wellcome Trust Center for Human Genetics, Oxford, UK). Genotype calling was performed automatically by the LightCycler 480 II software. Validation of genotyping results deviated from the wild-type cluster, analysis of samples that had failed (no amplification) the genotyping experiment, and cosegregation studies, were carried out by direct automated sequencing. Primers and PCR conditions are shown in Supplementary Material, Table S1. Sequencing was performed on an ABI Sequencer 3730 and data analyzed using Mutation Surveyor v.3.10.

In Series no.2, Sanger sequencing was used to screen for mutations in exon 13 of POLE, where POLE p.L424V is located, and in exon 11 of POLD1, where POLD1 p.S478N is located. Primers and PCR conditions are shown in Supplementary Material, Table S1. Sequencing was performed on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and data were analyzed using Sequencing Analysis v.5.1 and Variant Reporter v.1.1 (Applied Biosystems, Foster City, CA, USA).

**Loss of heterozygosity**

Microsatellites mapping close to POLE and expanding 1.356 Mb, D12S1723, D12S1628, D12S357 and D12S1638, were analyzed to assess LOH in DNA extracted from formalin-fixed paraffin-embedded tissue (10). Also, SNaPshot targeting the...
mutation p.L424V was used to assess LOH and to discriminate wild-type and mutated alleles. Primers and conditions are shown in Supplementary Material, Table S1. LOH was scored if the intensity of any allele was reduced by ≥50% relative to the other allele after taking account of the relative allelic intensities in paired constitutional DNA.

**KRAS, NRAS and BRAF mutation screening**

Analysis of KRAS mutations at codons 12 and 13 was performed using KRAS StripAssay (ViennaLab Diagnostics GmbH, Vienna, Austria), following manufacturer’s instructions. Exons 3 and 4 of KRAS, exons 2, 3 and 4 of NRAS, and BRAF V600E were assessed by direct automated (Sanger) sequencing. Primers, and PCR and sequencing conditions are available upon request.

**In silico prediction analysis**

In silico studies to assess the impact of amino acid substitutions (missense variants) on protein structure, function and evolutionary conservation were performed with SNPs3D, PolyPhen-2, SIFT and CONDEL algorithms (20–23).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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