Constitutional POLE variants causing a phenotype reminiscent of constitutional mismatch repair deficiency

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Abstract
Heterozygous POLE or POLD1 germline pathogenic variants (PVs) cause polymerase proofreading associated polyposis (PPAP), a constitutional polymerase proofreading deficiency that typically presents with colorectal adenomas and carcinomas in adulthood. Constitutional mismatch-repair deficiency (CMMRD), caused by germline bi-allelic PVs affecting one of four MMR genes, results in a high propensity for the hematological, brain, intestinal tract, and other malignancies in childhood. Non-malignant clinical features, such as skin pigmentation alterations, are found in nearly all CMMRD patients and are important diagnostic markers. Here, we excluded CMMRD in three cancer patients with highly suspect clinical phenotypes but identified in each a constitutional heterozygous POLE PV. These, and two additional POLE PVs identified in published CMMRD-like patients, have not previously been reported as germline PVs despite all being well-known somatic mutations in hypermutated tumors. Together, these five cases show that specific POLE PVs may have a stronger “mutator” effect than known PPAP-associated POLE PVs and may cause a CMMRD-like phenotype distinct from PPAP. The common underlying mechanism, that is, a constitutional replication error repair defect, and a similar tumor spectrum
INTRODUCTION

Three distinct mechanisms ensure accurate DNA replication before cell division. The DNA polymerases ε and δ (Pol ε, Pol δ) ensure high replication fidelity by the base selectivity of their active sites as well as their 3′-to-5′ exonuclease proofreading activity, which detects and excises misincorporated nucleotides. Post-replication, the DNA mismatch repair (MMR) system corrects errors that escape proofreading (Burgers & Kunkel, 2017; Ganai & Johansson, 2016; Jiricny, 2006; Rayner et al., 2016). Compromised polymerase proofreading and MMR deficiency have been observed in a variety of tumor types. MMR deficiency results from biallelic inactivation of one of four MMR genes (MLH1, MIM# 120436; MSH2, MIM# 609309; MSH6, MIM# 600259; PMS2, MIM# 600259) and is associated with the well-described mutator phenotype of microsatellite instability (MSI), an increased frequency of insertion and deletion mutations (indels) in short tandem repeats. MMR deficiency is observed in approximately 15% of colorectal and 26% of endometrial cancers, and at a lower frequency in other tumors (Ryan et al., 2019; Vilar & Gruber, 2010). Polymerase proofreading deficiency is caused by missense variants in the exonuclease domains of Pol ε (residues 268–471) and Pol δ (residues 304–517), which are encoded by POLE (MIM# 174762) and POLD1 (MIM# 174761) (Rayner et al., 2016). Approximately 2% of colorectal cancers and 7%–10% of endometrial cancers have POLE exonuclease domain PVs associated with a hyper-mutated phenotype with a tumor mutation burden (TMB) >10 mut/Mb (Barbieri & Scherbakova, 2017; Cancer Genome Atlas Research Network et al., 2013; Church et al., 2013, 2015; Shinbrot et al., 2014). Hypermutated stomach, pancreatic, and breast cancers, as well as high-grade gliomas with POLE PVs have also been found (Esern-Omaya et al., 2015; Shinbrot et al., 2014; Zou et al., 2014). Somatic POLD1 PVs are also associated with hyper-mutated tumors, albeit less frequently than POLE PVs (Rayner et al., 2016).

Although typically observed in neoplastic cells, constitutional MMR deficiency (CMMRD) and constitutional polymerase proofreading deficiency, called polymerase proofreading-associated polyposis (PPAP), are rare hereditary cancer predisposition syndromes (Palles et al., 2013; Wimmer et al., 2014). Over 200 CMMRD patients, most often caused by germline biallelic PVs in PMS2 or MSH6, have been published, and CMMRD is now a recognized recessively inherited, childhood cancer syndrome (MIM# 276300). The tumor spectrum includes hematological malignancies and brain tumors, as well as colorectal adenomas and carcinomas, and other Lynch syndrome-associated cancers. The median age at diagnosis of the first malignancy is <10 years (Wimmer et al., 2014). Nearly all CMMRD patients have characteristic, nonmalignant clinical features. Of these, multiple café-au-lait macules (CALMs) and other alterations of skin pigmentation are the most prevalent (Wimmer, Rosenbaum et al., 2017). The Care for CMMRD (C4CMMRD) consortium has integrated these features into a clinical scoring system to guide diagnostic workup for CMMRD in pediatric and young adult cancer patients (Wimmer et al., 2014).

Less than a decade ago, it was shown that germline PVs in the exonuclease domains of POLE and POLD1 cause PPAP, a dominantly inherited colorectal polyposis and cancer predisposition syndrome (Palles et al., 2013). Up to now, some 149 individuals from 55 unrelated families with PPAP have been reported (Table S1). Approximately half of these families (n = 28) have the recurrent POLE PV p.Leu424Val. Endometrial, ovarian, pancreatic, and breast cancers, as well as high-grade gliomas, have also been reported in patients with PPAP. The median age at the first malignancy was 43 years (range: 16–67 years) in 122 individuals carrying a POLE PV and was 41 years (range 21–64 years) in 25 individuals with a POLD1 PV.

Recently, two pediatric cancer patients have been reported with a clinical presentation highly suggestive of CMMRD but caused by constitutional POLE PVs (Lindsay et al., 2019; Wimmer, Beilken et al., 2017). Interestingly, the constitutional POLE PVs identified in these cases have not been reported to cause PPAP despite being known somatic mutations in hyper-mutated tumors. Here, we report three further cases with a CMMRD-like phenotype caused by constitutional POLE PVs not associated with PPAP but previously found as somatic mutations in ultra-mutated tumors (TMB >100 mut/Mb).

2 MATERIAL AND METHODS

2.1 Editorial policies and ethical consideration

All clinical and genetic analyses of the cases described here were performed as part of their diagnostic work-up. The patients/or their legal representative gave informed consent to all analyses and written informed consent to germline genetic testing. Written informed consent has been obtained from all patients and/or their legal representatives to publish clinical information and pictures of their skin pigmentation alterations.
2.2 | Tumor analysis

Tumor tissue fixation and sectioning, histology, and immunohistochemistry (IHC), as well as RNA extraction, DNA extraction, and MSI analysis, all followed standard diagnostic practices (please see Supporting Information for details).

Tumor DNA-methylation analysis used the Illumina Infinium EPIC/850 k BeadChip array (Illumina) according to the manufacturer’s instructions and using 250 ng of sample DNA. The unprocessed IDAT files were uploaded to the DKFZ/Heidelberg neuro classifier (https://www.molecularneuropathology.org/mmp) and subsequently compared to methylation data of a reference cohort in the Neuro methylation classifier v.11b4. Matching is obtained if the score is >0.9.

For whole-exome comparative tumor sequencing, germline and tumor DNA (500 ng) were fragmented to 300 bp using Covaris S2 (Agilent), and adaptor ligation was performed on a Sciclone G3 (Perkin Elmer) using KAPA HTP Library Preparation Kit (Roche). Exomes were enriched with SureSelectXT Clinical Research Exome kit (Agilent). Somatic single-nucleotide variants and small indels were called using GATK 4.1.7 best practices (Van der Auwera & O’Connor, 2020). In brief, sequencing reads were trimmed using bbduk v.38.26 (https://sourceforge.net/projects/bbmap/) and aligned to the human reference genome (hg19/GRCh37) using BWA v.0.7.15 mem (Li & Durbin, 2009). Aligned reads were filtered using GATK PrintReads and base quality scores were recalibrated using GATK bqsr. Somatic variant calling used GATK Mutect2 and somatic variant calls were filtered using GATK FilterMutectCalls. Variants were normalized with vt v.0.5772 normalize (Tan et al., 2015) and annotated using Ensembl Variant Effect Predictor v.104 vep (McLaren et al., 2016).

Mutational signatures were explored using the latest (as of June 2021) version of the R/Bioconductor package MutationalPatterns (Blokzijl et al., 2018). Briefly, count matrices were derived for all three different types of mutations (single base substitutions, double base substitutions, and indels) and their cosine similarity to the mutational signatures of the COSMIC reference signature collection v3.2 (Alexandrov et al., 2020) was calculated. COSMIC cosine similarity scores were normalized against a background cohort of 95 somatic whole-genome sequencing samples to account for distinct inter-signature cosine similarity distributions: Specifically, each cosine similarity was normalized by dividing the difference between them and the median cosine similarity by the interquartile range of cosine similarity values for that signature. For presentation, signatures were grouped by etiology.

2.3 | Germline/constitutional analyses

Sequencing and mutation analysis of germline DNA extracted from peripheral blood leukocytes (PBLs) followed standard diagnostic practices (please see Supporting Information for details). All variants identified are described in accordance with the Human Genome Variation Society (http://www.hgvs.org/mutnomen) guidelines. The following reference sequences were used, RefSeq NM_006231.4 (LRG_789) for POLE, and NM_000179.3 (LRG_219) for MSH6. The A of the ATG start codon is position c.1.

CMMRD-negative genetic diagnoses were confirmed using a sequencing-based MSI assay that can detect the low-frequency microsatellite length variants in DNA extracted from non-neoplastic PBLs of patients with CMMRD (Gallon et al., 2019). The assay was performed with adaptions as described in (Perez-Valencia et al., 2020), and an MSI score >2.00 was used to classify a patient as CMMRD-positive.

3 | RESULTS

An extensive description of the clinical course and follow-up of each case reported here can be found in Supporting Information.

3.1 | Case 1

Case 1 presented at the age of 4 years and 4 months with a large tumor (5.1 × 3.8 × 3.1 cm) in the right cerebellar hemisphere. On physical examination, the patient had one hypo- and multiple hyperpigmented skin spots distributed over the entire body (Figure 1a-d). More than six were reminiscent of CALMs and had a diameter over 0.5 cm. CALMs were absent in the non-related parents and in two healthy older siblings. Both parents were healthy with no history of cancer. A maternal aunt was diagnosed with colon cancer at the age of 30 years. The maternal grandfather died at the age of 35 years with leukemia (diagnosed at 29 years of age) and the maternal grandmother had Hodgkins’ lymphoma at the age of 65 years and non-Hodgkins lymphoma at the age of 66 years. The patient scored at least four points according to C4CMMRD criteria (Table 1) and, hence, was further evaluated for CMMRD.

Pathological analysis of the brain tumor, including genome-wide methylation (850K) analysis, using the Heidelberg classifier, showed it to be a WHO grade IV anaplastic medulloblastoma of subtype SHH (score 0.97) and subclass SHH A (score 0.82), with desmoplastic/nodular features and without amplification of MYC or MYCN (Figure S1A and S1B). Tumor MSI testing and immunohistochemical (IHC) staining of the four MMR proteins were performed due to the clinical suspicion of CMMRD. The tumor was microsatellite stable but showed loss of MSH6 expression in neoplastic cells. However, MSH6 expression was retained in non-neoplastic epithelial cells (Figure S1E). Staining for MLH1, MSH2, and PMS2 demonstrated regular nuclear expression in all cells (Figures S1C, S1D, and S1F).

Germline mutation analysis of MSH6 and the other MMR genes, as well as genes associated with hereditary medulloblastoma (PTCH1, SUFU, APC, BRCA2, PALB2, and TP53), revealed neither a (potentially) pathogenic single nucleotide change nor copy number aberration in any gene. In addition, a highly sensitive MSI assay, which detects increased MSI in non-neoplastic PBLs as a diagnostic hallmark of CMMRD (Gallon et al., 2019), excluded CMMRD. To further explore targeted treatment and the mechanisms leading to malignancy, extensive genetic profiling of the tumor was pursued. Whole exome sequencing revealed a TMB of 266 mut/Mb, consistent with...
an ultra-mutated phenotype. Among the somatic mutations were two MSH6 PVs p.(Glu846*) and p.(Glu847*) located in trans (Figure S2), explaining the loss of MSH6 expression in the neoplastic cells. Cosine similarity analysis using COSMIC mutational signatures (Alexandrov et al., 2013; Blokzijl et al., 2018), showed the mutational pattern of the tumor to best match SBS signatures 14, 15, and 20, as well as DBS signature 10 (Figures 2b and S3). All four COSMIC signatures are associated with MMR deficiency, reflecting the presence of somatic MSH6 PVs. However, SBS signatures 14 and 20 are specific for concurrent MMR deficiency and polymerase proofreading deficiency (Haradhvala et al., 2018; Hodel et al., 2018). The germline analysis was extended to include POLE and identified a heterozygous missense variant c.1381T>A, p.(Ser461Thr), affecting a highly conserved amino acid in its exonuclease domain. This variant is not reported in the background population (gnomAD), but was recently described as an ultra-mutated medulloblastoma and remarkable skin pigmentation alterations.

3.2 | Case 2

Case 2 was diagnosed with IDH1-wildtype glioblastoma (WHO IV) at the age of 29 years. At the age of 20 years, the patient had been diagnosed with a colorectal adenocarcinoma at the splenic flexure (G2 pT2), and multiple (50–60) colorectal adenomas (mainly of a tubulovillous histology, and several showed high-grade dysplasia). At the age of 28 years, the patient had a calcifying epithelioma/pilomatrixoma (0.5 × 1.5 × 1.4 cm) removed from his upper arm (Figure 1g,h). The patient reported that a similar cyst was removed in childhood. Physical examination of the patient at the age of 30 years showed multiple café au lait-colored skin spots distributed over the entire body and several skin papules and cysts on his forehead and trunk (Figure 1e,f). Their family history revealed that the maternal grandmother and a maternal great-uncle had colorectal cancer in their 70s, but no further cancer diagnoses were reported. Taken together, at the time of glioblastoma diagnosis the patient scored at least nine points according to C4CMMRD criteria (Table 1), raising clinical suspicion of CMMRD.

Previously, IHC staining of the MMR proteins MLH1, MSH2, and MSH6, as well as MSI testing, had indicated that both the tumor and non-neoplastic tissue of the resected colorectal cancer had normal MMR function. Moreover, PMS2 staining of both glioblastoma and colon tumor tissues, as well as gMSI testing of non-neoplastic PBLs according to Ingham et al. (2013), showed no evidence of MMR deficiency in any tissue. Retrospectively, MSI analysis of PBLs according to Gallon et al. (2019) also ruled out CMMRD. Performing mutation analysis of the adenomatous polyposis genes APC, MUTYH, NTHL1, MLH3, POLE, POLD1, and the four MMR genes, we identified the heterozygous variant c.890C>T, p.(Ser297Phe), in the exonuclease domain of POLE as the only potential PV. This variant has not been reported in association with PPAP but has been found as a somatic mutation in different tumor types with high TMBs (Campbell et al., 2017; Rayner et al., 2016) and mutational signatures of Pol ε proofreading deficiency (León-Castillo et al., 2020; Shinbrot et al., 2014). Furthermore, a structural analysis showed that amino acid p.Ser297 interacts with an exonuclease catalytic site residue, and is hence likely to alter the active site conformation (Briggs & Tomlinson, 2013). Germline DNA analysis of the healthy parents showed POLE p.(Ser297Phe) is de novo in the patient and, hence,
| **C4CMRRD feature**                                                                 | **Case 1**                                                                 | **Case 2**                                                                 | **Case 3**                                                                 | **Wimmer et al. (2017)** | **Lindsay et al. (2019)** |
|----------------------------------------------------------------------------------|---------------------------------------------------------------------------|---------------------------------------------------------------------------|---------------------------------------------------------------------------|--------------------------|--------------------------|
| **Malignancies/pre-malignancies:**                                               |                                                                           |                                                                           |                                                                           |                          |                          |
| Carcinoma from the LS spectrum at age <25 years                                   |                                                                           | CRC (adenocarcinoma splenic flexure), 20y, (3)                           | CRC (rectal adenocarcinoma), 13y, (3)                                     |                          |                          |
| Multiple bowel adenomas at age <25 years and absence of APC/MUTYH mutation(s)    |                                                                           | Adenomatous polyposis (50–60 mainly tubulovillous adenomas several with high-grade dysplasia in large intestine, numerous small polyps in ileum), 20y, (3) | adenomatous polyposis, 13, (3)                                          |                          |                          |
| WHO grade III or IV glioma at age <25 years                                       |                                                                           | glioabloma (WHO IV, IDH1-wildtype), 29y, (0)                             |                                                                          |                          |                          |
| Any malignancy at age <18 years                                                   |                                                                           | Medulloblastoma (anaplastic with desmoplastic/nodular features, SHH A subgroup with somatic TP53 mutation), 4y, (1) |                                                                            |                          | Medulloblastoma (anaplastic, non-WNT/non-SHH), 5y, (1) |
| **Additional features:**                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Clinical sign of NF1 and/or ≥2 hyper- and/or hypo-pigmented skin alterations Ø>1 cm in the patient | >5 CALM & other hyper- & hypo-pigmented skin alteration, (2)               | Multiple CALM & other hyperpigmented skin alterations, (2)               | Multiple CALM, (2)                                                       |                          |                          |
| Carcinoma from LS spectrum before the age of 60 in 1st-, 2nd- or 3rd-degree relative |                                                                           |                                                                           | 6 CALM, (2)                                                             | >100 CALM & other hyperpigmented skin alterations (2) |
| Pat. aunt: CRC, 30y (1); mat. grandfather: leukemia 29y (0); mat. grandmother: Hodgkins lymphoma and non-Hodgkins lymphoma 66y, (0) |                                                                           |                                                                           |                                                                           |                          |                          |
| Pat. aunt: CRC, 30y (1); mat. grandmother & mat. great-uncle: CRC, >60y, (0)     |                                                                           |                                                                           |                                                                           |                          |                          |
| Pat. grandmother: breast ca. >70y, (0)                                            |                                                                           |                                                                           |                                                                           |                          |                          |
| Tectal plate glioma, 11y; intramuscular venous malformation (probably congenital)  |                                                                           |                                                                           |                                                                           |                          |                          |
| Unilateral complex renal cyst, tibial osteochondroma                                |                                                                           |                                                                           |                                                                           |                          |                          |
| **Pilomatricoma(s) in the patient**                                               |                                                                           | One malignant calcifying epithelioma/pilomatrixoma (similar cyst removed in childhood), (1–2) | One pilomatricoma, (1)                                                  | Three pilomatricomas, (2) |
| **Total C4CMRRD scoring points**                                                  | 4                                                                        | ≥9                                                                       | 8                                                                         | 9                        | 5                        |
| **Additional clinical features with no C4CMRRD scoring points:**                 |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
| Medulloblastoma: 266 mut/Mb, SBS14, SBS15, SBS20, DBS10                         |                                                                           |                                                                           |                                                                           |                          |                          |
| Glioblastoma: 320 mut/Mb (FoundationOne), 463 mut/Mb,                            |                                                                           |                                                                           |                                                                           |                          |                          |
| CRC: 169 mut/Mb, SBS10b                                                           |                                                                           |                                                                           |                                                                           |                          |                          |
further supported its causal role for the patient’s phenotype. Retrospective mutation analysis of the glioblastoma with the FoundationOne™ (Foundation Medicine) gene panel revealed 54 genomic alterations, equaling a TMB of 320 mut/Mb and two somatic MSH6 PVs, c.3173-1G>T and p.Arg1172fs. Additional retrospective exome sequencing of the tumor by Morgenstern et al. (2021), identified COSMIC SBS mutational signatures 14 and 15 as the best match for the tumor mutation pattern (see Morgenstern et al., 2021, study ID ICI.33). Both signatures are associated with MMR deficiency and SBS signature 14 is also associated with concurrent polymerase proof-reading deficiency, which is consistent with the combined constitutional POLE p.(Ser297Phe) PV and two somatic MSH6 PVs, assuming these are located in trans, in the tumor.

### 3.3 Case 3

Case 3 presented at 13 years of age with a rectal mass of 6.0 × 5.0 × 2.0 cm and numerous (>100) adenomatous polyps in the colon, the largest measuring 3.0 × 2.0 × 1.9 cm (Figure 1j). The final staging of the rectal tumor was T3N1M0, consistent with stage IIIB rectal carcinoma. On physical examination, the patient showed several CALMs over the legs, abdomen, back and one on her face (Figure 1i). A review of her records revealed that she had been followed in the neurosurgery clinic due to the incidental discovery of an unbiopsied tectal plate glioma two years before her diagnosis of rectal cancer (Figure 1k). During the time of her evaluation for colorectal cancer, she was also seen by an orthopedic surgeon for a 10-year-long history of a right shoulder mass that was surgically removed and found to be an intramuscular venous malformation (Figure 1l). The family history of this only child was reviewed in detail. The paternal grandmother had breast cancer diagnosed in her seventies or eighties, but there were no cases of colorectal cancer or young-onset tumor. According to C4CMMRD criteria, the patient scored 8 points (Table 1) and was suspected of having CMMRD.

Hematoxylin and eosin stains of the rectal carcinoma were consistent with a diagnosis of invasive poorly differentiated adenocarcinoma with mucinous and signet ring cells. IHC staining of the MMR proteins demonstrated regular nuclear expression of MLH1, MLH2, MSH6, and PMS2. Retrospectively performed MSI testing of PBLs according to Gallon et al. (2019) ruled out CMMRD as the most likely diagnosis. The patient was evaluated for a cancer predisposition syndrome using mutation analysis of 154 genes included in the Comprehensive Hereditary Cancer Panel of Blueprint Genetics, in constitutional DNA extracted from PBLs. A heterozygous POLE variant, c.1331T>A, p.(Met444Lys), was identified. POLE p.(Met444Lys) affects a highly conserved amino acid in the Pol ε exonuclease domain and has been described as a somatic mutation consistently associated with a high TMB (Campbell et al., 2017; Johnson et al., 2017) with the typical mutational signature of Pol ε proofreading deficiency. Retrospectively performed exome sequencing revealed a TMB of 169 mut/Mb for the rectal carcinoma and a high cosine similarity to the Pol ε proofreading deficiency SBS signature 10b (Figure S3).
Germline DNA analysis showed that this POLE variant is absent in the healthy mother. The father was not available for testing.

4 | DISCUSSION

The three cases described here add to two published case reports (Lindsay et al., 2019; Wimmer, Beilken, et al., 2017) for a total of five patients with constitutional Pol ε proofreading deficiency and a CMMRD-like phenotype. All five patients fulfilled C4CMMRD scoring criteria to raise clinical suspicion of CMMRD (Table 1), suggesting that suspected CMMRD patients in whom the diagnosis cannot be genetically confirmed should be tested for constitutional POLE PVs. Analysis of POLD1 should also be considered as, like POLE, variants have been found as somatic driver mutations in hyper-mutated tumors and in the germline of PPAP patients (Table S1). Whilst the causative POLE PVs were confirmed to be de novo in two of these five cases (the father was not available for genetic testing in the other three), offering predictive testing of siblings may be advisable to take into account the very low risk of germ-cell mosaicism in one of the parents. Similarities with CMMRD were also apparent in molecular analyses of the medulloblastoma and glioblastoma of Case 1 and Case 2, respectively. Both had somatic PVs in MSH6, illustrating that somatic MMR deficiency can occur in a background of constitutional polymerase proofreading deficiency. As expected, the complete loss of replication error repair in the tumors produced high TMBs characterized by COSMIC mutational signature 14 (our data and data from Morgenstern et al., 2021). Almost all CMMRD-associated brain tumors have somatic POLE or POLD1 PVs, which also lead to ultramutated tumors (Bouffet et al., 2016; Shlien et al., 2015) that have COSMIC SBS signature 14 and 20, respectively (Haradhvala et al., 2018; Hodel et al., 2020). Hence, Case 1 and Case 2 demonstrate that CMMRD-associated and constitutional Pol ε proofreading deficiency-associated tumors may have indistinguishable mutational signatures of concurrent MMR and polymerase proofreading deficiency. This should be taken into account when performing genome or exome sequencing of tumors to direct treatment and identification of germline genetic defects as is advocated for pediatric brain tumors (Campbell et al., 2017; Gröbner et al., 2018). The clinical and molecular similarities between CMMRD and these patients, as well as a common constitutional defect in replication error repair, also provide a good rationale for these patients to be monitored according to protocols proposed for CMMRD (Durno et al., 2017; Tabori et al., 2017; Vasen et al., 2014).

The phenotypes of these five cases appear to be distinct from and more severe than PPAP. For example, although Cases 2 and 3, and the first reported case in the literature (Wimmer, Beilken, et al., 2017) had colorectal tumors consistent with a polyposis phenotype, these were diagnosed at exceptionally young ages. Furthermore, Case 1 and the case reported by Lindsay et al. (2019) were diagnosed with a pediatric medulloblastoma, which does not fall into the known tumor spectrum of PPAP but is seen in approximately 5% of CMMRD patients (Wimmer, Rosenbaum et al., 2017). All of the cases reported here have other features, such as CALMs and pilomatrixomas used in the C4CMMRD scoring system to identify potential CMMRD patients. Case 2 also had an intramuscular venous
malformation, and venous malformations of the brain have been proposed as an additional diagnostic criterion for CMMRD (Shiran et al., 2018). Complementing their distinct phenotypes, all five cases have constitutional **POLE** PVs that have not previously been reported as germline variants in PPAP despite being known somatic mutations in hyper-mutated (TMB >10 mut/Mb) tumors (Figure 3 and Table S1). This suggests that these **POLE** PVs may have an exceptional constitutional penetrance.

Indeed, in vivo and in vitro yeast experiments have shown that different polymerase exonuclease domain variants equivalent to PVs in human Pol ε can have very different impacts on replication mutation rate (Barbari et al., 2018; Hamzaoui et al., 2020; Xing et al., 2019). Of note, the highest mutation rates exceeded those observed in exonuclease domain negative yeast strains, suggesting that mutation rate is not solely determined by exonuclease activity (Hamzaoui et al., 2020; Xing et al., 2019). For example, it has been shown that the yeast equivalent to human Pol ε p.(Pro286Arg), Pol2 p.(Pro301Arg), can more efficiently extend 3’ terminus mismatches, with incorporation of the mismatch into the extension product, and more efficiently bypass hairpin structures than exonuclease negative Pol2 (Xing et al., 2019). Galati et al. (2020) recently developed two mouse models with constitutional **POLE** variants equivalent to human p.(Pro286Arg) and p.(Ser459Phe). Survival differed significantly despite both models developing tumors with classic Pol ε proofreading deficiency-related mutational signatures and high TMBs. In particular, **POLE**p.(Pro286Arg) homozygous mice were not viable whereas **POLE**p.(Ser459Phe) homozygotes were, and, consistent with this, **POLE**p.(Pro286Arg/+)* heterozygotes had a more severe phenotype than **POLE**p.(Ser459Phe/+)* heterozygotes. However, the less penetrant p.(Ser459Phe) variant had a significantly greater in vitro reduction of exonuclease activity (Galati et al., 2020), providing further evidence for the diverse impact of **POLE** PVs and showing that in vitro studies do not necessarily reflect the observations of in vivo models.

Functional data on the **POLE** PVs identified in the three cases with CMMRD-like phenotypes described here, and the two previous cases from the literature (Lindsay et al., 2019; Wimmer, Beilken et al., 2017), are limited and, as the results of Galati et al. (2020) and others (Barbari et al., 2018; Hamzaoui et al., 2020; Mur et al., 2020) show, in vitro assays should be interpreted with caution due to different impacts of equivalent polymerase variants between humans and model organisms. However, the functional impact of these and some PPAP-associated variants have been assessed using human tumor sequence data, allowing the comparison of mutation rates between them (Table S1). Using a tumor mutational signature-based **POLE** variant scoring method, **POLE** p.(Ser297Phe) (Case 2), p.(Val411Leu) (Wimmer, Beilken et al., 2017), p.(Met444Lys) (Case 3), and p.(Ala456Pro) (Lindsay et al., 2019) all scored highly (**POLE**-score 4-6) whereas, in contrast, the most frequent PPAP-associated germline **POLE** p.(Leu424Val), had a lower score (**POLE**-score 3) (León-Castillo et al., 2020). In agreement, Campbell et al. (2017) observed that most (5/7) tumors with the PPAP-associated p.(Leu424Val) variant were not hyper-mutated, whereas the variants from the five cases with CMMRD-like phenotypes were all associated exclusively with hyper-mutated tumors. These tumor data...
suggest that PPAP-associated POLE PVs may have a weaker "mutator" effect than those found only in hyper- or ultra-mutated tumors and in the CMMRD-like cases described here. However, it should be noted that assessing variant impact using tumor sequence analysis is complicated by the natural history of the tumors: Tumors may have multiple polymerase variants and other endogenous or exogenous exposures, such as MMR deficiency, that influence mutation rate and signature (Campbell et al., 2017). Furthermore, PPAP-associated variants may be predominantly associated with hyper-mutated tumors, such as POLE p.(Pro436Ser) (Hamzaoui et al., 2020; Spier et al., 2015) in the study of Campbell et al. (2017), where all (5/5) tumors carrying this variant were hyper-mutated (Figure 3 and Table S1). Therefore, identification of more patients and the development of novel functional assays and models may be needed to clarify the genotype–phenotype relationship of constitutional POLE variants.

In the literature, we found additional patients with constitutional POLE PVs and potentially CMMRD-like phenotypes (Figure 3 and Table S1). Most notably, a patient with a constitutional POLE p.(Pro436Arg) variant diagnosed with a colorectal adenocarcinoma and anaplastic astrocytoma (WHO grade III glioma) at age 17 years has been reported. This patient scores at least five points according to C4CMMRD criteria based on their malignancies alone (other clinical features were not disclosed) (Galati et al., 2020; Shuen et al., 2019). This variant has not been observed in PPAP and, in tumor sequence analyses, was associated exclusively with hyper-mutated tumors (Campbell et al., 2017) and had a high POLE-score (León-Castillo et al., 2020), agreeing with the genotype-phenotype relationship observed in our cases. Three more patients with early-onset cancer and constitutional POLE variants score at least three points according to C4CMMRD criteria. One patient had colorectal adenomas aged <25 years, CALMs, and family members affected with a Lynch syndrome-spectrum cancer aged <60 years, associated with a constitutional POLE p.(Glu277Gly) variant (Rosner et al., 2018). The second had a colorectal cancer at age 23 years and a constitutional POLE p.(Asn363Lys) variant (Hamzaoui et al., 2020; Vande Perre et al., 2019), and the third had a glioblastoma aged 16 years and a constitutional POLE p.(Asp368Asn) variant (Hamzaoui et al., 2020). However, these three patients have PPAP pedigrees, and their POLE variants have not been observed in tumor analyses or were found in isolated tumors making interpretation of their functional impact uncertain (Figure 3 and Table S1). Therefore, they may represent extremes of the PPAP phenotypic spectrum, possibly caused by genetic or environmental modifiers. Another patient carrying a POLE p.(Tyr458Asn) variant was diagnosed with a colorectal cancer aged 25 years and has an affected daughter diagnosed with multiple colorectal adenomas at age 17 years (Shickl et al., 2021). Despite not raising suspicion for CMMRD according to C4CMMRD criteria, these are very early-onset phenotypes. POLE p.(Tyr458Asn) has not been described in tumors or in a PPAP family to our knowledge (Figure 3 and Table S1), hence this father and his daughter may also fit the suggested genotype-phenotype relationship of POLE variants.

In conclusion, the three cases presented here, and two previously published case reports (Lindsay et al., 2019; Wimmer, Beilken et al., 2017) may represent a distinct pediatric cancer syndrome caused by constitutional polymerase proofreading PVs that are not associated with PPAP but are associated with hyper-mutated tumors. Regardless, the clinical similarities of these cases to CMMRD suggest constitutional polymerase proofreading deficiency should be considered as a differential diagnosis to CMMRD, and these patients should, based on the known phenotype thus far, be managed according to clinical guidelines for CMMRD.

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Conflict of Interests
Richard Gallon is named as the inventor on patents held by Cancer Research UK Commercial Partnerships covering the markers used in the MSI assay used in this study (patent ID: PCT/GB2017/052488, published 1 March 2018; and PCT application number: PCT/GB2019/052148, unpublished, filing date 31 July 2019). All other authors have no conflicts of interest to declare.

Data availability statement
Data available on request due to privacy/ethical restrictions.

Web resources
POLYPHEN-2 (http://genetics.bwh.harvard.edu/pph2), SIFT (http://sift.bii.a-star.edu.sg), MutationTaster (http://www.mutationtaster.org), Align GVGD (http://agvgd.hci.utah.edu/agvgd_input.php), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and gnomAD (https://gnomad.broadinstitute.org/).

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Additional supporting information may be found in the online version of the article at the publisher’s website.

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