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A Novel Deleterious Variant and a Founder Effect in Four New Families of *MBD4*-Associated Neoplasia Syndrome Recruited Over a Period of 20 Years

Inês Querido[1] | Carla Pinto[1,2,3] | Patrícia Arinto[1,2] | Andreia Brandão[1] | Catarina Santos[1,2] | Manuela Pinheiro[1] | Joana Guerra[1,2] | João Silva[1,4] | Ana Peixoto[1,2] | Manuel R. Teixeira[1,2,5,6]

[1]Cancer Genetics Group, IPO Porto Research Center (CI-IPOP)/RISE@CI-IPOP (Health Research Network), Portuguese Oncology Institute of Porto (IPO Porto)/Porto Comprehensive Cancer Center, Porto, Portugal | [2]Department of Laboratory Genetics, Portuguese Oncology Institute of Porto (IPO Porto)/Porto Comprehensive Cancer Center, Porto, Portugal | [3]Department of Pathological, Cytological and Thanatological Anatomy, School of Health, Polytechnic Institute of Porto, Porto, Portugal | [4]Department of Medical Genetics, Portuguese Oncology Institute of Porto (IPO Porto)/Porto Comprehensive Cancer Center, Porto, Portugal | [5]Department of Pathology and Molecular Immunology, School of Medicine and Biomedical Sciences (ICBAS), University of Porto, Porto, Portugal | [6]European Reference Network on Genetic Tumour Risk Syndromes (ERN GENTURIS), Porto, Portugal

Correspondence: Manuel R. Teixeira (manuel.teixeira@ipoporto.min-saude.pt)

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ABSTRACT

DNA glycosylases play a crucial role in DNA repair mediated by the base excision repair (BER) pathway, and alterations in these enzymes have been associated with hereditary cancer predisposition. Recently, germline biallelic loss-of-function variants in *MBD4* were shown to be responsible for a novel autosomal recessive multi-tumor predisposition syndrome, provisionally denominated as *MBD4*-associated neoplasia syndrome and characterized by the association of adenomatous polyposis, colorectal cancer, and acute myeloid leukemia (AML). Here, we studied the *MBD4* gene in five individuals from four families affected by adenomatous polyposis and AML, who had been referred for genetic counselling at a single institution over a period of approximately 20 years. All patients with this phenotype presented homozygous deleterious germline variants in *MBD4*, of which one is a founder variant recurrent in three of the families, and another variant has not been previously described in the literature. Our work allowed a molecular diagnosis for these families and significantly contributes to expanding the knowledge about this emerging syndrome caused by *MBD4* constitutional deficiency.

1 | Introduction

The human genome is prone to the action of endogenous and exogenous damaging agents, which induce alterations in DNA. These mutations constitute a threat to genetic integrity and are commonly associated with human diseases, including cancer [1]. However, DNA repair systems play a crucial role in restoring genomic damage, thus preserving genetic information and guaranteeing its faultless transmission [2]. The base excision repair (BER) pathway is a primary mechanism for the correction of nonbulky DNA lesions in a damage-specific manner [2, 3]. Among the key players in BER are DNA glycosylases, which are responsible for the recognition and excision of damaged bases. In humans, the BER pathway involves 11 subtypes of DNA

glycosylases, and alterations in these enzymes and concomitant malfunction of BER lead to mutagenesis and compromise genomic integrity, which may contribute to cancer development [2–4].

It is well established that biallelic deleterious germline variants in the *MUTYH* gene, coding the MutY homolog DNA glycosylase, are responsible for the autosomal recessive cancer predisposition syndrome *MUTYH*-associated polyposis (MAP), mainly characterized by an increased risk of developing adenomatous polyposis and colorectal cancer (CRC) [5–8]. The identification of this syndrome put the BER pathway in the spotlight for a role in hereditary cancer, which led to the search for new polyposis and CRC predisposing genes. Subsequently, biallelic deleterious germline variants in the *NTHL1* gene, coding the Nth-like DNA glycosylase 1, were demonstrated to be the cause of *NTHL1* tumor syndrome, an autosomal recessive disorder mainly associated with increased susceptibility for adenomatous polyposis, CRC, and other neoplasias, such as breast cancer [6, 9, 10].

More recently, the gene encoding the methyl-CpG binding domain protein 4 (MBD4), another DNA glycosylase of the BER pathway, was also identified as a cancer predisposing gene. Deactivation of *MBD4* due to biallelic loss-of-function germline variants was shown to be responsible for hereditary predisposition to adenomatous polyposis, CRC, and extracolonic neoplasms, mainly early-onset acute myeloid leukemia (AML) and uveal melanoma [11–14]. To date, a total of 11 individuals, from eight distinct families, have been described, of whom 10 presented colorectal polyps, seven also developed AML, and two also presented uveal melanoma. *MBD4* constitutive deficiency is therefore the cause of a rare autosomal recessive multi-tumor predisposition syndrome, which has been provisionally denominated as *MBD4*-associated neoplasia (MANS) by Palles et al. [12].

In this study, we set out to explore *MBD4* germline variants in patients affected simultaneously with adenomatous polyposis and AML, who had been referred to a single tertiary oncology institution for genetic counseling and germline genetic testing over a period of approximately 20 years.

2 | Materials and Methods

2.1 | Patients, Samples and DNA Extraction

This study included five individuals, from four families, affected with adenomatous polyposis and AML who had been referred to the Portuguese Oncology Institute of Porto between 2004 and 2023 for genetic counseling and germline genetic testing. Notably, these four families were the only ones, out of a total of 343 families with polyposis evaluated during this period, that demonstrated the simultaneous occurrence of polyposis and leukemia. All five patients were counseled by one of the authors (M.R.T.), who noticed the peculiar association between polyposis and AML and initially ordered genetic testing for the genes known at the time to be associated with hereditary predisposition to polyposis. The four index patients tested negative for deleterious germline variants in the *APC* and *MUTYH* genes.

In order to study the *MBD4* gene, we used archived samples from the index patients, in which DNA had been previously extracted from peripheral blood using standard procedures. In addition, whenever possible, relatives of the index patients were also studied for the presence of the germline variant detected in the family. The study was approved by the IPO Porto Ethics Committee (reference number 122/023) and all individuals enrolled signed informed consent for diagnostic genetic testing and for research.

2.2 | *MBD4* Germline Variant Analysis

Screening of germline variants in the coding regions (exons 1–8) and flanking splice junctions of the *MBD4* gene was performed by Sanger sequencing. For this purpose, DNA was amplified using different primer sets (Table S1; details of amplification conditions are available upon request). The sequencing reaction was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's recommendations. Furthermore, the products were analyzed using a 3500 Genetic Analyzer (Thermo Fisher Scientific).

Subsequently, *MBD4* was integrated into the hereditary cancer gene panel routinely used in our laboratory using next-generation sequencing (NGS). In more detail, specific capture probes targeting the *MBD4* coding regions and flanking splice sites were added to the TruSight Hereditary Cancer panel (Illumina, San Diego, CA, USA). Library preparation was carried out according to the manufacturer's instructions, and sequencing was performed on the NextSeq 550 platform (Illumina Inc.). Sequencing alignment and variant analysis were performed using a bioinformatics pipeline previously validated by our group [15]. Positive samples by Sanger sequencing were used as controls.

All *MBD4* variants were described according to the Human Genome Variation Society (HGVS) recommendations, using NM_001276270.2 as the reference sequence.

2.3 | Haplotype Analysis

To evaluate if the recurrent *MBD4* germline variant c.1544-1G>T identified in three families resulted from founder effects in the population, identical-by-descent (IBD) haplotype and phylogenetics analyses were performed. The index patients of families B and D (not enough DNA was available from the index patient of family C) and two controls were genotyped using the CytoScan HD Array (Thermo Fisher Scientific). The resulting genotype data was converted to PLINK files (PED/MAP) and VCF files, using an *in-house* python script and PLINK 2.0 [16]. SNPs on chromosome 3 were phased using BEAGLE 4.1 [17] and IBD haplotype analysis was performed using the BEAGLE Refined IBD algorithm [18]. The lengths of the shared haplotype segments were estimated by the distance between the two last shared SNPs flanking the variant. Phylogenetic networks were reconstructed based on the median joining algorithm [19] using PopART v1.7 [20], and phylogenetic trees based on the genetic distance were constructed

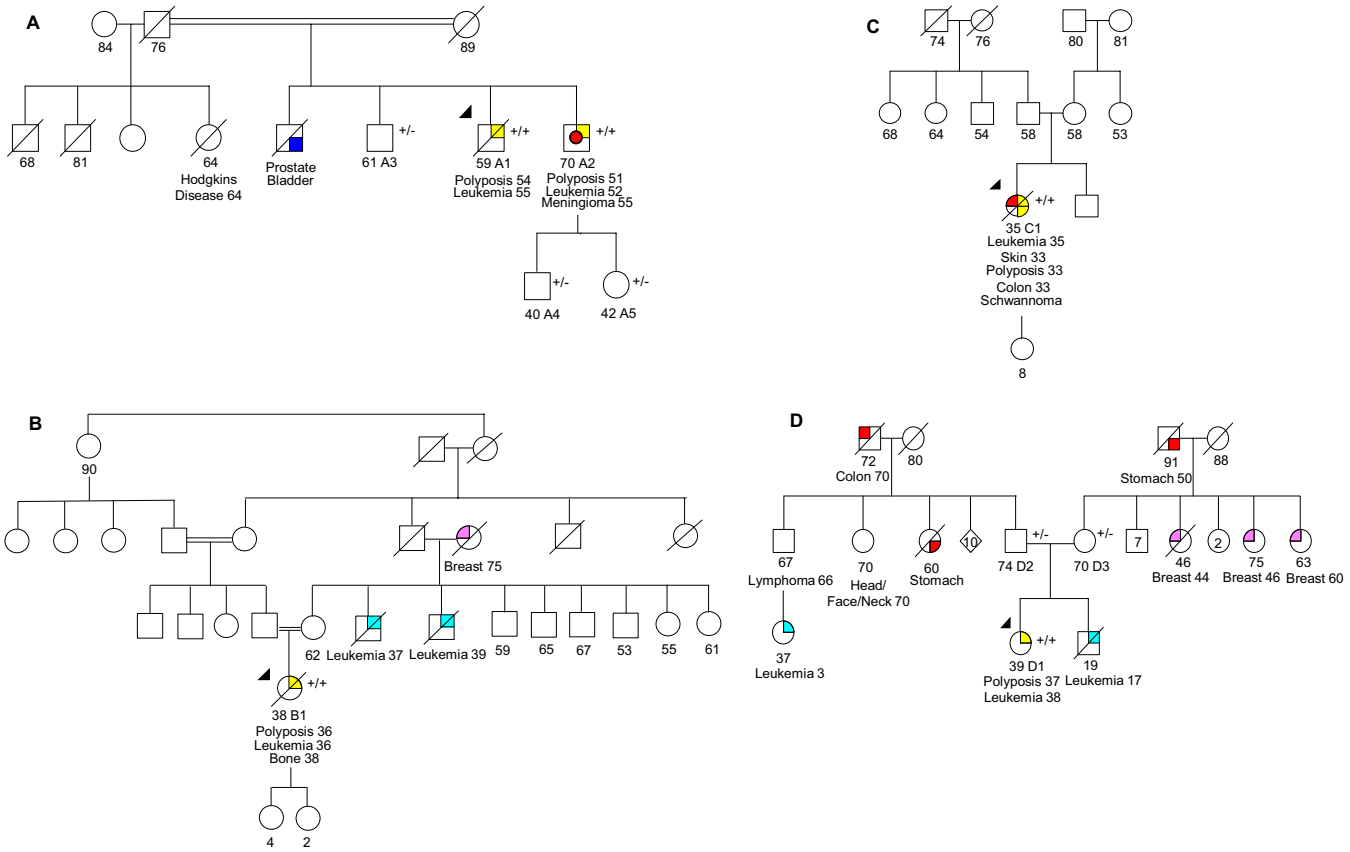

FIGURE 1 | Pedigrees of the four families with biallelic deleterious germline variants in the *MBD4* gene. (A) Family presenting the novel variant NM_001276270.2:c.[538del];[538del] p.[(Thr180ProfsTer35)];[(Thr180ProfsTer35)]. (B–D) Families presenting the splicing variant NC_000003.11(NM_001276270.2):c.[1544-1G>T];[1544-1G>T]. +/+ represents biallelic variant carrier; +/− represents heterozygous variant carrier. Color symbol: Blue lower right corner—prostate cancer; pink upper left corner—breast cancer; red central circle—brain tumor; red lower right corner—gastric cancer; red upper left corner—colorectal cancer; turquoise upper right corner—leukemia; yellow lower right corner—skin tumor; yellow upper right corner—polyposis. Numbers displayed below each symbol represent the individual's age at last follow-up, while numbers alongside each neoplasm indicate age at diagnosis. [Colour figure can be viewed at wileyonlinelibrary.com]

using clustalw2 [21] and visualized using the FigTree (v.1.4.4) program (available from http://tree.bio.ed.ac.uk/software/figtree/). All analyses were carried out using GRCh37/hg19 chromosomal positions.

3 | Results

Searching for germline variants in *MBD4* by Sanger sequencing revealed that all five patients included in this study present deleterious homozygous germline variants in this gene, which allowed us to establish the molecular diagnosis of the syndrome in these four families.

Patient A1 was a man who was diagnosed with adenomatous polyposis at the age of 54 years old and underwent a total colectomy (since the procedure was performed at an external institution, detailed information regarding the number and histological characteristics of the polyps are not available). One year later, he was diagnosed with AML with dysplasia and normal karyotype. His first genetic consultation was in September 2006, and he eventually passed away at 59 years old. His brother, patient A2, was diagnosed with polyposis at 51 years old, presenting more than 70 polyps, the majority tubular or tubulovillous, and only 4 hyperplastic. He was also diagnosed with AML (French-American-British (FAB) subtype M2) 1 year later and with meningioma at 55 years old. In 2016, he underwent a prophylactic total colectomy. These patients had consanguineous parents, but they are not sure exactly how they were related (Figure 1). Patient A1 was shown to harbor a previously unreported *MBD4* germline variant in homozygosity, the c.538del p.(Thr180ProfsTer35), which is predicted to result in a premature stop codon in exon three (Figure 2). An archived DNA sample isolated from peripheral blood from patient A2 (the affected sibling of patient A1) showed the presence of the *MBD4* germline variant previously detected in his brother, but in heterozygosity (Figure 2). Upon reviewing the patient's medical records, we noticed that he had undergone allogeneic hematopoietic stem cell transplantation (HSCT) with a healthy sibling as the donor (relative A3, who is presumed to be a heterozygous variant carrier). In order to properly analyze the presence of the *MBD4* germline variant in patient A2, we used DNA isolated from a buccal swab sample, which revealed that this patient also presents the *MBD4* variant in homozygosity (Figure 2).

Patient B1 was a woman diagnosed at 36 years old with adenomatous polyposis and with AML presenting dysplasia and

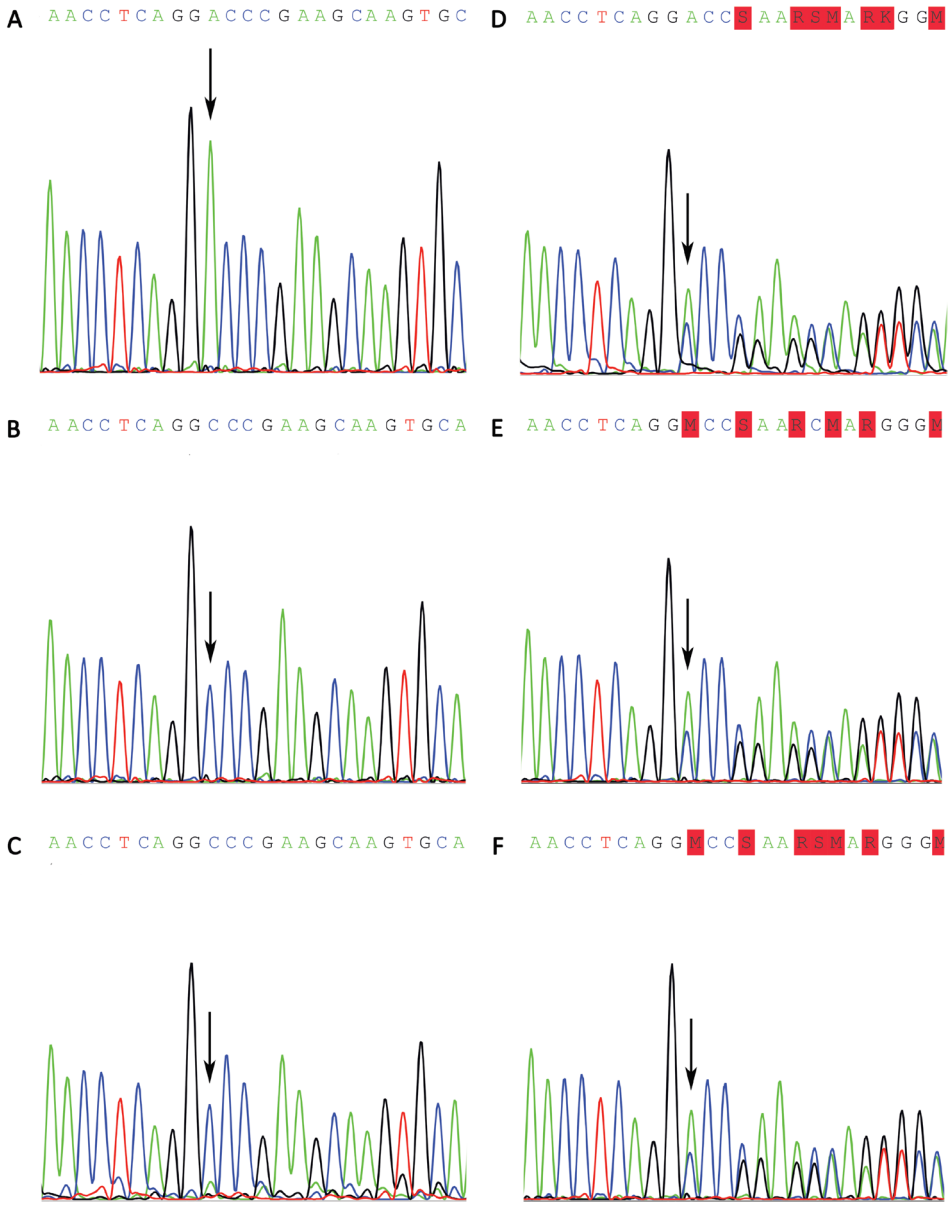

FIGURE 2 | Sanger sequencing electropherograms of the *MBD4* NM_001276270.2:c.538del variant; (A) Normal sequence (homozygous wildtype); (B) Homozygous variant in patient A1; (C) Heterozygous variant in patient A3 (peripheral blood sample from patient A2—bone marrow recipient of relative A3); (D) Homozygous variant in patient A2 (buccal swab sample); (E) Heterozygous variant in relative A4; (F) Heterozygous variant in relative A5. The position of the variants is indicated by arrows. [Colour figure can be viewed at wileyonlinelibrary.com]

normal karyotype. Two years later, she presented a benign bone tumor, for which the differential diagnosis between giant cell tumor or aneurismal bone cyst could not be established. This patient had her first genetic consultation in November 2010 and underwent a total colectomy later that year, during which more than 100 polyps were observed, all of which were tubular/tubulovillous. She eventually passed away at 38 years old. The parents of this patient were consanguineous, and she had two deceased maternal uncles, both diagnosed with leukemia at the age of 37 and 39, respectively (Figure 1). This patient was shown to present the *MBD4* germline variant c.1544-1G>T in homozygosity (Figure 3), also described in the literature as c.1562-1G>T using the NM_001276271.2 as reference. Patient C1 was a woman diagnosed with adenomatous polyposis (with a total of 39 tubular/tubulovillous adenomas and 11 hyperplastic polyps), CRC and a perineal basal cell carcinoma (BCC) at the age of 33 and with AML 2 years later. Moreover, she presented a schwannoma, for which it was not possible to access information about the exact age of onset. This patient had her first genetic consultation in July 2011. She passed away at the age of 35 years old, and no other relevant family history or consanguinity was known (Figure 1). This patient was also shown to be homozygous for the *MBD4* germline variant c.1544-1G>T. The archived DNA sample from this patient was highly degraded; however, the presence of the variant was suspected by Sanger sequencing and subsequently confirmed by next-generation sequencing (NGS) after *MBD4* was included in the routine testing panel (Figure 3). Patient D1, whose first genetic consultation was in October 2021, is a woman who was diagnosed at 37 years old with adenomatous

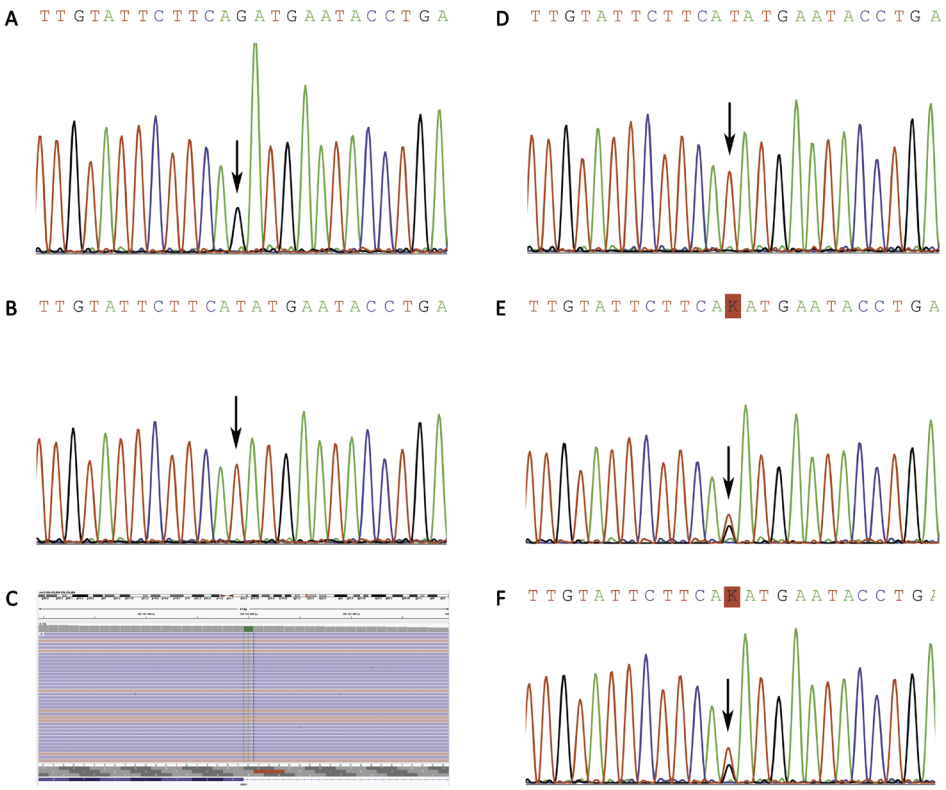

FIGURE 3 | Sanger sequencing electropherograms and Integrative Genomics Viewer (IGV) alignment of the *MBD4* NC_000003.11(NM_001276270.2):c.1544-1G>T variant; (A) Normal sequence (homozygous wildtype); (B) Homozygous variant in patient B1; (C) Homozygous variant in patient C1; (D) Homozygous variant in patient D1; (E) Heterozygous variant in relative D2; (F) Heterozygous variant in relative D3. The position of the variants is indicated by arrows. [Colour figure can be viewed at wileyonlinelibrary.com]

polyposis, and one of the polyps exhibited a small focus of intramucosal carcinoma. One year later, she was also diagnosed with AML presenting dysplasia and normal karyotype. This patient underwent a total colectomy in 2022, during which more than 50 polyps were identified, all of which were tubular/tubulovillous. After the colectomy, eight additional tubular/tubulovillous polyps were excised from the rectum. She has a deceased brother who was diagnosed with leukemia at 17 years old and passed away 2 years later. In addition, she presents a family history of several cancers in second- and third-degree relatives (Figure 1), but no consanguinity was known. This patient also presents the *MBD4* germline variant c.1544-1G>T in homozygosity (Figure 3). Despite belonging to apparently unrelated families, patients B1, C1, and D1 present the same *MBD4* germline variant, which affects splicing, and according to Rodrigues et al. [22], analysis of RNA-seq demonstrated that it promotes the skipping of exon 7, and the frameshift consequence disrupts the glycosylase domain, being classified as likely pathogenic in ClinVar (ID 1700251).

Additionally, whenever possible, we studied first-degree relatives of the index patients who accepted genetic testing for the presence of the germline variant previously detected in the family. Both the children of patient A2 (relatives A4 and A5) and the parents of patient D1 (relatives D2 and D3), who do not present any personal history of cancer, were shown to harbor the *MBD4* germline variant previously detected in patient A2 (Figure 1A) and in patient D1 (Figure 1D), respectively, but in heterozygosity (Figures 2 and 3).

Finally, IBD haplotype and phylogenetic analyses were conducted on two of the carriers of the recurrent *MBD4* germline variant c.1544-1G>T and two controls to evaluate the existence of a founder effect in the population. The IBD analysis identified two main haplotypes among the *MBD4* variant carriers, extending from 0.9 Mb (H1: chr3: 129045906-129987881) to 1.4 Mb (H2: chr3: 128651195-130025249; Figure 4). Phylogenetic analysis of these haplotypes supported a greater genetic similarity between variant carriers compared to non-carriers (Figure 4B,D). The haplotype reconstruction also unveiled a shared core homozygotic segment of approximately ~178 Kb flanking the variant (chr3: 129045906-129223869) among all carriers, which was not detected by the IBD analysis, probably due to its small size. This conserved region was not shared with the non-carriers (Figure 4E). Collectively, these findings support a founder origin for this variant. Additionally, to investigate the broader genetic variability flanking the *MBD4* germline variant c.1544-1G>T, we reconstructed a phylogenetic tree for an extended flanking region of approximately 2.5 Mb (Figure 4F). A reduced genetic variability between the haplotypes of each carrier compared to the non-carriers was observed. Interestingly, patient B1 showed considerably greater genetic distance from the other patients, likely due to the consanguinity in the family.

4 | Discussion

MBD4 acts as a T and U glycosylase and removes the mismatched bases that arise from the deamination of 5mC and C

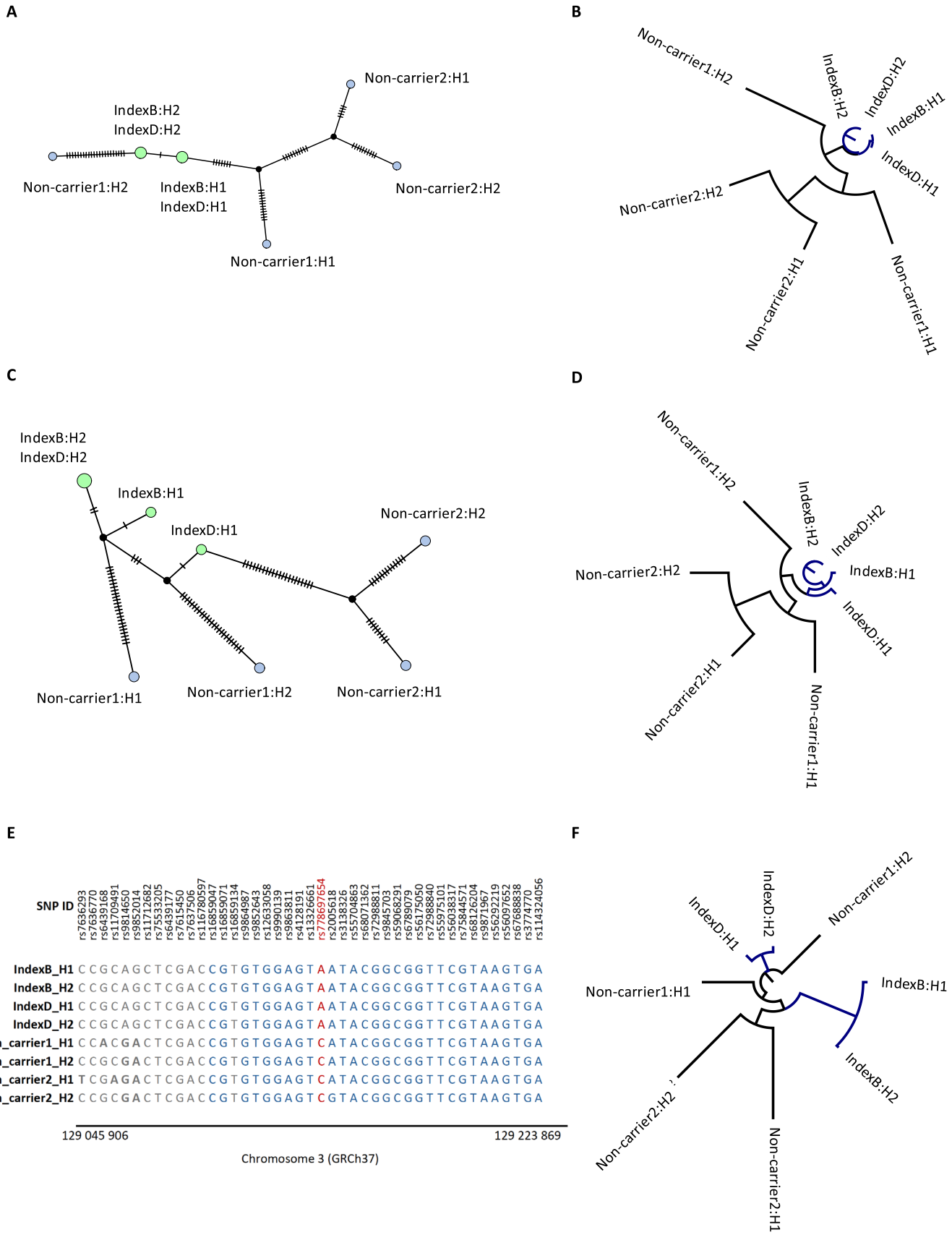

FIGURE 4 | Legend on next page.

FIGURE 4 | Haplotype and phylogenetics analysis of the *MBD4* c.1544-1G>T variant. (A, B) Median joining network and phylogenetic tree reconstruction of haplotype 1 identified by IBD analysis (H1: chr3: 129045906-129987881; ~0.9 Mb). In the network, each circle represents a haplotype, and circle size is proportional to the number of individuals carrying the specific haplotype. (C, D) Median joining network and phylogenetic tree reconstruction of haplotype 2 identified by IBD analysis (H2: chr3: 128651195-130025249; ~1.4 Mb). (E) Sequence alignment of the genotyped SNPs from the CytoScan HD Array comprising the core shared haplotype, approximately 178 Kb in size, flanking the variant (chr3: 129045906-129223869). The *MBD4* c.1544-1G>T variant is highlighted in red, while the allelic variation in non-carriers is shown in bold. Intronic positions are colored in blue, and intergenic are in grey. (F) Phylogenetic tree of an approximately 2.5 Mb region flanking the *MBD4* germline variant c.1544-1G>T (ch3: 128651195-131157799). [Colour figure can be viewed at wileyonlinelibrary.com]

in CpG sites, preventing the further formation of C>T mutations upon DNA replication [23, 24]. Therefore, this enzyme contributes to maintaining genomic stability and is presumed to act as a tumor suppressor [1]. Beyond that, it seems to be involved in other crucial biological processes, such as apoptosis and epigenetic regulation, but these mechanisms still remain poorly established [25–28]. Defects in the *MBD4* gene have been demonstrated to lead to an accumulation of C>T transitions along the genome, which can contribute to tumorigenesis via genomic instability [29, 30]. Similarly to other DNA glycosylases of the BER system, namely *MUTYH* and *NTHL1*, constitutive loss of *MBD4* function has recently been associated with inherited cancer predisposition. Deleterious biallelic germline variants in this gene were shown to be responsible for MANS [12], a novel autosomal recessive hereditary cancer syndrome, which has so far only been reported in eight families and is mainly characterized by an increased susceptibility to develop adenomatous polyposis, CRC and other neoplasms, mainly early-onset AML [11–14].

In the present work, we describe a set of four new families presenting individuals affected with adenomatous polyposis and AML, which are homozygous for deleterious germline variants in the *MBD4* gene, constituting one third of the total of families with MANS currently described (Table 1). It is important to highlight, regarding families A and D, that it was possible to study the offspring of patient A2 and the parents of patient D1, who were shown to be heterozygous carriers of the respective germline variants. This result demonstrates that the variant is not *de novo* in individual D1 (it was inherited from both parents) and confirms the Mendelian transmission of the variant in family A (since the offspring of a homozygous individual must be obligate heterozygous carriers).

The phenotypes of the patients included in our study are consistent with those described in the literature, although there is limited available information [11–14]. Nevertheless, this evidence indicates that the main clinical manifestations of MANS appear to be predisposition to adenomatous polyposis, CRC, and AML. In addition, it is possible to observe that the affected individuals, as it has been previously documented [12, 13], frequently present benign lesions, namely schwannoma and meningioma. However, this study is the first to describe a BCC and a benign bone lesion in the context of MANS, thus contributing to expand the tumor spectrum of the syndrome, although further studies are necessary to validate these associations.

The *MBD4* variant c.1544-1G>T has been previously described in heterozygosity associated with predisposition to uveal melanoma [31, 32]. Furthermore, this variant has only been previously reported once in patients with biallelic inactivation, namely in two siblings presenting AML, one of whom was also affected with colorectal polyps and CRC [11]. Notably, these individuals carried this variant in compound heterozygosity; hence, the three families we here present are the first to be reported with this variant in homozygosity. Furthermore, IBD haplotype and phylogenetics analysis of two of the carriers of this variant revealed shared haplotypes extending from 0.9 to 1.4 Mb flanking the variant. Additionally, a smaller core haplotype was identified surrounding the variant in the carriers, which was not conserved in controls. These findings support a founder effect for this variant rather than multiple independent occurrences, similarly to other variants found in cancer-associated genes reported across several populations, including in the Portuguese population [32–35]. Moreover, as recombination events over time typically break down larger haplotypes into smaller segments, the identification of the small core haplotype surrounding the *MBD4* variant c.1544-1G>T, along with the variant's presence in other populations [11, 12], suggests an ancient origin for this variant.

On the other hand, the *MBD4* variant c.538del p.(Thr180ProfsTer35) we found in family A has, to our knowledge, not been previously described in the literature. Following the American College of Medical Genetics and Genomics (ACMG) guidelines, we classify it as likely pathogenic, since it consists of a frameshift variant that most likely generates a premature stop codon in the central interdomain region, which is predicted to result in a truncated MBD4 protein lacking the glycosylase domain (PVS1) and presents an extremely low population frequency in population databases (PM2).

The molecular diagnosis of the syndrome in these families enables genetic testing for the patients' relatives, which allows presymptomatic diagnosis and may eventually contribute to the development of surveillance programs and possible prophylactic measures for biallelic variant carriers. Additionally, it allows non-carriers to be exempted from unnecessary intensified cancer screening, as their cancer risk resembles that of the general population. Based on the described phenotypes and in accordance with the National Comprehensive Cancer Network (NCCN) guidelines, clinical surveillance concerning MANS should primarily focus on the high risk for colorectal polyposis and early-onset AML for biallelic variant carriers, which includes regular colonoscopies from age 18 to 20 years and complete blood count (NCCN Guidelines, 2023). Moreover, considering the recurrence of other malignancies and benign lesions, regular clinical monitoring should be recommended for these individuals. Of note, the scarce available data indicate that heterozygous variant carriers do not present an increased susceptibility for the most

TABLE 1 | Clinicopathological characteristics of the patients presenting biallelic *MBD4* deleterious germline variants.

Family	Patient	Gender	Malignancies (age of onset[a])	Benign tumors (age of onset[a])	*MBD4* germline variants		Publication
					DNA (NM_001276270.2)	Protein effect prediction	
1	EMC-AML-1	M	Polyposis; AML (33)	—	c.[1681_1683del]; [1681_1683del]	p.[(His561del)]; [(His561del)]	(11)
2	WEHI-AML-1	F	AML (31)	—	c.[939dup]; [1544-1G>T]	p.[(Glu314ArgfsTer13)];[?]	
	WEHI-AML-2	F	AML (34); Polyposis; CRC (40)	—			
3	—	M	Polyposis; CRC (24); Lymphoma (28); AML (30)	—	c.[1273C>T]; [1670T>A]	p.[(Arg425Ter)]; [(Leu557Ter)]	(14)
	—	F	Polyposis (33); AML (35); Papillary thyroid cancer (44)	Schwannoma (44)			
4	D:II-1	M	Polyposis (36); AML (49)	—	c.[612_615del]; [612_615del]	p.[(Ser205ThrfsTer9)]; [(Ser205ThrfsTer9)]	(12)
5	CRDFF-292-1:II-3	M	Polyposis, UVM (53)	Liver and small renal cysts (53)	c.[939dup];[939dup]	p.[(Glu314ArgfsTer13)]; [(Glu314ArgfsTer13)]	
6	CRDFF-336-1:II-1	F	OvGCT (12); Polyposis (39)	—			
	CRDFF-336-2:II-2	M	Polyposis (39)	—			
7	DB1-70:II-3	F	Polyposis (35); UVM (38, 45); Upper GITVA (49)	Meningioma (41); DCIS, Schwannoma (50)	c.[939dup];[1670T>A]	p.[(Glu314ArgfsTer13)]; [(Leu557Ter)]	(12)
8	—	M	Polyposis (32); AML (37)	Schwannoma (34)	c.[1517G>A]; [1517G>A]	p.[(Arg506Gln)]; [(Arg506Gln)]	(13)
9	A1	M	Polyposis (54); AML (55)	—	c.[538del];[538del]	p.[(Thr180ProfsTer35)]; [(Thr180ProfsTer35)]	This study
	A2	M	Polyposis (51); AML (52)	Meningioma (55)			
10	B1	F	Polyposis, AML (36)	Giant cell tumor/aneurismal bone cyst[b] (38)	c.[1544-1G>T]; [1544-1G>T]	p.[?];[?]	
11	C1	F	Polyposis, CRC, BCC (33); AML (35)	Schwannoma			
12	D1	F	Polyposis[c] (37); AML (38)	—			

Abbreviations: AML—acute myeloid leukemia; BCC—basal cell carcinoma; CRC—colorectal cancer; DCIS—ductal carcinoma in situ of the breast; F—female; GITVA—gastrointestinal tract tubulovillous adenoma; M—male; OvGCT—ovarian granulosa cell tumor, UVM—uveal melanoma.

[a]Whenever available.

[b]The differential diagnosis between the two hypotheses could not be established.

[c]One of the polyps had a small focus of intramucosal adenocarcinoma.

common MANS manifestation, including polyposis and AML. However, some reports suggest that monoallelic variant carriers may present an increased risk for uveal melanoma [31, 32, 36]. Although the five MANS patients we here describe did not present uveal melanoma, two of the eight cases previously described presented that phenotype. Therefore, regular ophthalmologic exams are recommended both for heterozygous carriers and for homozygous or compound heterozygous variant carriers (NCCN Guidelines, 2023).

In addition, the identification of *MBD4* germline variants may have an influence on therapeutics. For example, regarding AML patients, the selection of a sibling as a bone marrow donor for allogenic HSCT should ideally be guided by prior genetic testing. Furthermore, it is worth noting that MANS patients present a high mortality rate, which highlights the importance of exploring alternative treatment approaches in order to eventually improve their outcomes. Some studies reported that patients with *MBD4*-deficient uveal melanoma, which typically harbor a heterozygous *MBD4* deleterious germline variant accompanied by somatic loss of the second normal allele, exhibit a good response to immune checkpoint inhibitors [32, 36]. Immunotherapy has been approved by the US Food and Drug Administration (FDA) for the treatment of patients with metastatic CRC displaying MSI or high tumor mutational burden [37, 38] and, although patients harboring BER deficiency per se are not formally eligible for this type of treatment, there are some case reports, at least in MAP patients, demonstrating a good clinical response [39, 40]. Therefore, we speculate that MANS patients may possibly represent good candidates for immune checkpoint inhibitors therapy, since these tumors have also been described to harbor a hypermutated nature [11–14], thus probably being more immunogenic and responsive to immunotherapy. However, further investigation is required to evaluate this possibility.

By describing five new individuals, from four families, presenting *MBD4* germline variants in homozygosity, our work significantly contributes to expanding the knowledge about this emerging syndrome, as well as providing new features about *MBD4* constitutional deficiency, including the expansion of the syndrome tumor spectrum, the identification of the novel *MBD4* deleterious variant c.538del, and the demonstration of a founder effect for the c.1544-1G>T variant. Our findings strongly suggest that *MBD4* should be included in next generation sequencing multi-gene panels used to study patients with personal and/or family history of colorectal polyposis, especially when accompanied by AML or uveal melanoma.

Author Contributions

Conceptualization: M.R.T. Methodology: I.Q., C.P., A.B., and P.A. Formal analysis: I.Q., C.P., A.B., and P.A. Writing – original draft preparation: I.Q. Writing – review and editing: I.Q., C.P., A.B., P.A., C.S., M.P., J.G., J.S., A.P., and M.R.T. Supervision: M.R.T. Funding acquisition: M.R.T. All authors have read and agreed to the published version of the manuscript.

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Ethics Statement

This study was conducted in accordance with the Declaration of Helsinki and approved by the IPO Porto Ethics Committee (reference number 122/023).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Peer Review

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/cge.70014.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.