



Confirmation of *FZD5* implication in a cohort of 50 patients with ocular coloboma

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Abstract

Defects in optic fissure closure can lead to congenital ocular coloboma. This ocular malformation, often associated with microphthalmia, is described in various clinical forms with different inheritance patterns and genetic heterogeneity. In recent times, the identification of an increased number of genes involved in numerous cellular functions has led to a better understanding in optic fissure closure mechanisms. Nevertheless, most of these genes are also involved in wider eye growth defects such as micro-anophthalmia, questioning the mechanisms controlling both extension and severity of optic fissure closure defects. However, some genes, such as *FZD5*, have only been so far identified in isolated coloboma. Thus, to estimate the frequency of implication of different ocular genes, we screened a cohort of 50 patients affected by ocular coloboma by using targeted sequencing of 119 genes involved in ocular development. This analysis revealed seven heterozygous (likely) pathogenic variants in *RARB*, *MAB21L2*, *RBP4*, *TFAP2A*, and *FZD5*. Surprisingly, three out of the seven variants detected herein were novel disease-causing variants in *FZD5* identified in three unrelated families with dominant inheritance. Although molecular diagnosis rate remains relatively low in patients with ocular coloboma (14% (7/50) in this work), these results, however, highlight the importance of genetic screening, especially of *FZD5*, in such patients. Indeed, in our series, *FZD5* variants represent half of the genetic causes, constituting 6% (3/50) of the patients who benefited from a molecular diagnosis. Our findings support the involvement of *FZD5* in ocular coloboma and provide clues for screening this gene during current diagnostic procedures.

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Introduction

The human eye development is a complex and well-conserved vertebrate embryological process. Broadly, it becomes visible during the 4th week of development from an initial interaction between the optic vesicle (derived from neuroepithelium) that will evolve in a double-layered optic cup, and the lens placode (derived from the surface ectoderm), which will in the meantime invaginate to form the lens vesicle [1, 2]. During this asymmetric process, a transient opening on the ventral face of the optic cup appears and allows the vascular and nervous systems to get through; it will finally close around the 7th week of embryonic life [3, 4]. This morphogenetic process occurs according to three different axes (proximal–distal, dorsal–ventral and nasal–temporal), involving many signalization pathways and a complex and intricated genetic regulation [1, 5].

Congenital ocular anomalies result from a defect in the molecular and cellular mechanisms of eye formation. For example, those affecting the entire ocular globe can lead to the severe spectrum of anophthalmia and microphthalmia (absence and reduced growth of the ocular globe, respectively) with a prevalence of 1–3 per 10,000 births [6]. Ocular coloboma consists in a defect of optic fissure closure [1–3]. The closure process requires first a correct alignment of the fissure margins, ensured by a correct patterning along the three axes of the optic cup [1]. Then, the fusion phenomenon can start at the midpoint to progress both distally and proximally, thanks to multiple processes: changes in cell morphology at the optic fissure, basement membrane dissolution and cell adhesion, apoptosis phenomena, all these interacting with periocular mesenchyme cells [1, 3, 4]. Given the optic fissure location, coloboma is de facto located in the inferonasal quadrant, potentially affecting any structure from the iris to the optic nerve [3, 7]. As a consequence, the phenotype may vary from normal vision to blindness forms [3]. In addition, ocular coloboma can be unilateral or bilateral, isolated or associated with other ocular malformations such as microphthalmia and anophthalmia (M/A) or with extraocular features in syndromic forms, such as CHARGE syndrome [MIM#214800] or Baraitser-Winter syndrome (BRWS) [MIM#243310] as well in several chromosomal aberrations [3, 7]. Moreover, inheritance varies from sporadic to familial cases with autosomal dominant, autosomal recessive or X-linked inheritance patterns reported. Worldwide prevalence ranges from 2 to 14 per 100,000 births causing 20% of childhood visual impairment [8]. Causes are multiple, from primary (congenital) to secondary, such as consequence of surgery, or environmental forms. Distinction is drawn between ocular coloboma and “atypical” coloboma resulting from iris defects whose genetic cause is different (mainly variants in *PITX2*, *FOXC1* and *PAX6*) [2, 3]. The large majority of the 30 genes described as implicated in ocular

coloboma are syndromic and non-syndromic M/A genes [1, 6, 9]. This clinical overlap is often entitled with the term “MAC” spectrum (M/A and Coloboma).

Interestingly, two genes have been so far identified in patients with isolated ocular coloboma: *FZD5* [MIM*601723] and a recurrent variant in *ACTG1* [MIM*102560] [10, 11]. *FZD5* (frizzled class receptor 5) has been involved in a single dominant form of coloboma, after whole-exome sequencing (WES) was performed in a large family, identifying an ultra-rare disease-causing variant in 13 individuals with either non-syndromic bilateral chorioretinal or iris coloboma ($n = 10$), unilateral optic disc (OD) abnormality ($n = 1$), or without any ocular phenotype ($n = 2$), considered as non-penetrant allele [10]. *FZD5* encodes for the FZD5 protein, which is a Wnt receptor that mediates canonical and non-canonical Wnt-signaling pathways [12]. The frameshift variant c.656delCinsAG [p.(Ala219Glufs*49)] detected in *FZD5* was shown to produce a truncated protein lacking the transmembrane domain (TMD), but with a conserved extracellular cysteine-rich Wnt-binding domain (CRD) [10]. The authors first showed that a disruption in the Wnt-FZD5 signaling pathway leads to a defect in optic fissure closure in humans, as already demonstrated in animal models [10, 13, 14]. Regarding *ACTG1*, it was formerly identified as the BRWS responsible gene [15]. Interestingly, a de novo *ACTG1* variant c.209C>T [p.(Pro70Leu)] was detected in two unrelated patients with bilateral iris and chorioretinal coloboma with no extraocular BRWS feature [11].

Thus, to identify the underlying genetic cause in a cohort of 50 patients affected with coloboma of unknown etiology, we performed a targeted resequencing approach using a 119 ocular developmental genes panel.

Materials and methods

Cohort description

This study was designed in compliance with the tenets of the Helsinki Declaration. Informed consent was obtained from all individuals included in this study.

A cohort of 50 patients with a clinical diagnosis of unilateral or bilateral coloboma in a familial ($n = 11$) or sporadic inheritance pattern ($n = 39$) was analyzed on a targeted NGS panel of 119 genes involved in various ocular defects. This targeted resequencing panel contains different categories of ocular developmental genes (Suppl.data1). In particular, it includes all the coloboma-associated genes described to date, as well as all the genes involved in overlapping phenotypes such as micro-anophthalmia (MAC spectrum) or anterior segment dysgeneses responsible of iris defects that can mimic iris coloboma. Indeed, among these 50 patients, 11 were displaying bilateral coloboma

associated with unilateral (4/11) or bilateral (7/11) microphthalmia and 11 patients were presenting with a coloboma affecting only the iris. Extraocular features were observed in 11 out the 50 patients for which no association was diagnosed as part of a known syndrome. Clinical and molecular data are summarized in Table 1. Additional array CGH was performed in patients with associated features such as intellectual disability and/or abnormalities (Table 1).

Targeted NGS panel analysis

The 50 patients' cohort was screened by means of a customized NGS panel including 119 ocular developmental genes (Suppl.data1). DNA was extracted from blood using the MagnaPure system (Roche Applied Science, Germany). Capture probes were designed with SureDesign (Agilent, USA). A library of all coding exons and intron-exon boundaries was prepared using the SureSelect XT HS and XT Low Input Enzymatic Fragmentation Kit and the SureSelect XT HS Target Enrichment System for Illumina Paired-End Multiplexed Sequencing Library (Agilent, USA) following the manufacturer's instructions. Further sequencing was performed on a NextSeq500 platform (Illumina Inc., CA). Sequence alignment was performed with BWA 0.7.10, picard-tools-2.18.23, elprep4 (Indel realignment, base recalibration). Then, the variant calling was made with GATK-3.3 (HaplotypeCaller) and VarScan2.3.7. Annotations were made with SNPEff-4.3 with additional information from gnomAD, ClinVar, and dbSNP151.

All variants were confirmed by Sanger sequencing. Sequence variants were numbered with the Adenine of the ATG initiation codon considered as the first nucleotide. GenBank references were: *FZD5*: NM_003468.4, *MAB21L2*: NM_006439, *PTCH1*: NM_000264, *RARB*: NM_000965, *RBP4*: NM_006744, and *TFAP2A*: NM_003220.

All the novel variants described in this study have been submitted to ClinVar database.

Assessment of pathogenicity

Variants were classified according to the ACMG guidelines [16]. All "Pathogenic", "likely pathogenic" and "of unknown significance" (VUS) variants were reported in this study. "Pathogenic" and "likely pathogenic" variants were considered as disease-causing variants when familial segregation of the variant was in accordance with the mode of inheritance. Of note, no *FZD5* transcript analysis could be performed.

Results

This exhaustive genetic analysis revealed seven disease-causing variants, respectively in *RARB*, *MAB21L2*,

TFAP2A, *RBP4* and *FZD5*, as well as one variant of uncertain significance in *PTCH1*, in 8 out the 50 probands (Table 1).

Likely pathogenic variants in *FZD5*

Three different *FZD5* likely pathogenic variants, never described before, were detected in three unrelated families with dominant inheritance. They were considered as disease-causing variants. Clinical and molecular data are detailed in Table 2.

Patient 1: sporadic bilateral non-syndromic coloboma

Patient 1 (ADN120143) was a 10-year-old girl, the first child of unrelated Algerian and Moroccan parents with no familial history of ocular malformation (apart from a right isolated convergent squint in the father, without coloboma). She was diagnosed with a right iris, choroidal and OD coloboma adjacent to the macula and a left choroidal and OD coloboma at 10 months of life during an ophthalmological examination for a right convergent squint. Neurocognitive development and growth parameters were unremarkable. She had no other malformation and her clinical examination showed no abnormality including the absence of distinctive craniofacial features. A heterozygous frameshift variation **c.820del** [p.(Leu274Cysfs*73)] was detected in *FZD5* (Suppl.data2). This variation was never reported in the literature and was absent from gnomAD. Complete segregation pattern could not be established due to the unavailability of the DNA's father. Following the ACMG guidelines [16], the variant is considered as likely pathogenic (PVS1, PM2). The ClinVar accession number for this variant is SCV000998820.

Patient 2: sporadic bilateral syndromic coloboma

Patient 2 (ADN140259) was a 43-year-old woman, the fourth out of six siblings, born from unrelated Algerian parents, herself mother of four children. Ocular familial history was unremarkable. She was diagnosed at birth with a right OD coloboma and a left iris and OD coloboma associated with a cortical cataract causing blindness. She suffers from bilateral congenital deafness with cochlear malformations (no further information available) and neurocognitive difficulties (trouble in learning, memorization, concentration). Cerebral MRI showed a postero-inferior and internal herniation of the left eyeball in contact with the optic nerve. Array CGH found no chromosomal imbalance. A heterozygous in-frame variant **c.1081_1082insGAA** [p.(His361delinsArgAsn)] was identified in *FZD5*. This variation was never reported in the literature and was absent from gnomAD. This in-frame codon insertion happens in

Table 1 Clinical and molecular data of the 50 coloboma affected patients.

Patients	Case	Reference	Sex	Age (years)	Ocular phenotype	Extraocular features	Familial history	Array CGH	119 genes panel
Without microphthalmia	1	ADN120143	F	10	R iris, choroidal, and optic disc coloboma; L choroidal and optic disc coloboma			Yes	<i>FZD5: c.820del, p.(Leu274Cysfs*73)</i>
	2	ADN140259	F	43	R optic disc coloboma; L iris and optic disc coloboma with cortical cataract	Bilateral congenital deafness, neurocognitive difficulties		Yes	<i>FZD5: c.1081_1082insGAA p.(His361delinsArgAsn)</i>
	3	SG170145	F	26	Bilateral iris and large chorioretinal coloboma, with R posterior cataract		Mother with bilateral papillary coloboma	Yes	<i>FZD5: c.1181_1246del p.(Asn394_Gly415del)</i>
	4	SG161597	M	3	Bilateral chorioretinal coloboma and R iris coloboma			No	
	5	ADN090126	M	12	R iris coloboma	Developmental delay		Yes	
	6	ADN090149	M	11	L iris coloboma	Hypospadias	Paternal cousin with coloboma and intellectual disability	Yes	
	7	ADN090193	F	10	Bilateral iris coloboma		Consanguinity	No	
	8	ADN100036	M	10	R iris coloboma with lens opacity			No	
	9	ADN110042	M	11	Unilateral iris coloboma			No	
	10	ADN120128	M	12	Bilateral iris coloboma			No	
	11	ADN120145	M	39	Bilateral iris and chorioretinal coloboma			No	
	12	SG182291	F	9	Bilateral chorioretinal coloboma			No	
	13	ADN120231	M	40	Bilateral coloboma with hyperopia			No	
	14	SG172356	M	2	Bilateral iris and chorioretinal coloboma			No	
	15	ADN130023	F	37	Bilateral optic nerve coloboma		Nephew with bilateral iris coloboma and cataract	No	
	16	ADN150083	M	6	Bilateral iris coloboma, with unilateral hyperopia	Diaphragmatic hernia		Yes	<i>RARB: c.872A > T, p.(His291Leu)</i>
	17	ADN150091	F	6	bilateral chorioretinal coloboma with L microcornea and myopia			No	
	18	SG181901	F	2	Bilateral iris coloboma; R chorioretinal and papillary coloboma		Mother with R iris and chorioretinal coloboma	No	
	19	ADN180127	M	2	Bilateral iris and retinal coloboma			Yes	
	20	SG060149	F	41	Ocular coloboma			No	
	21	ADN190153	M	5	Bilateral chorioretinal coloboma			Yes	
	22	SG190593	M	6	Bilateral iris and chorioretinal coloboma			Yes	
	23	SG080744	F	15	R iris coloboma; L macular and optic disc coloboma			No	<i>PTCH1: c.2500T > G, p.(Leu834Val)</i>
	24	SG080781	M	11	Bilateral iris coloboma	Bilateral single transverse palmar crease		No	
	25	SG091554	M	16	Bilateral optic nerve coloboma		Consanguinity	No	
	26	SG102070	M	19	Bilateral iris coloboma			No	
	27	SG102327	M	75	Bilateral retinal coloboma			No	<i>RBP4: c.569-1G > A, p.?</i>
	28	SG110094	F	9	R iris and chorioretinal coloboma		Deceased sister of a unilateral retinoblastoma	No	
	29	SG131110	M	8	Bilateral iris, chorioretinal and optic disc coloboma		Mother with bilateral keratoconus, cleft palate, unilateral renal agenesis, mitral valve disease	No	
	30	SG131574	M	6	Bilateral iris coloboma		Maternal half-brother with R iris coloboma, mother with cataract	No	
	31	SG132126	M	8	Bilateral iris coloboma			No	
	32	SG140040	M	31	Bilateral coloboma			Yes	
	33	SG141923	M	17	Bilateral coloboma			No	
	34	SG151978	M	23	Bilateral iris and retinal coloboma			No	
	35	SG170755	F	8	Iris and chorioretinal coloboma	Autism spectrum disorder	Sister with iris coloboma and learning difficulties	Yes	
	36	ADN060077	F	16	Bilateral iris and chorioretinal coloboma	Renal asymmetry		No	

Table 1 (continued)

Patients	Case	Reference	Sex	Age (years)	Ocular phenotype	Extraocular features	Familial history	Array CGH	119 genes panel
	37	ADN110045	M	16	R coloboma	Labio-palatal cleft and mild deafness		Yes	
	38	ADN150024	F	9	L iris and chorioretinal coloboma			No	
	39	SG190263	M	34	Bilateral iris and chorioretinal coloboma			No	
With microphthalmia	40	LGN120039	F	30	Bilateral colobomatous microphthalmia	Intellectual disability, autistic features, craniofacial dysmorphism, scaphocephaly	Father with isolated bilateral colobomatous microphthalmia	Yes	<i>MAB21L2: c.1A>C, p.(Met1?)</i>
	41	ADN140207	F	6	Bilateral iris and chorioretinal coloboma with microphthalmia		Consanguinity	No	
	42	SG191977	F	27	Bilateral optic disc coloboma, microcornea and microphthalmia		Father and brother affected with optic disc coloboma	No	
	43	SG191278	F	38	Bilateral coloboma with microphthalmia		Maternal uncle with microphthalmia	No	
	44	SG060418	F	15	R iris coloboma; L chorioretinal coloboma, with bilateral microphthalmia			Yes	
	45	SG060983	M	23	Bilateral iris and chorioretinal coloboma with microphthalmia	Mild intellectual disability		Yes	
	46	SG061227	M	51	Bilateral iris coloboma with cataract and R microphthalmia			No	
	47	SG062099	F	14	Bilateral optic disc coloboma with R microphthalmia		Father with large papillary excavation	No	
	48	SG072054	M	12	Bilateral optic disc coloboma with L microphthalmia			Yes	
	49	SG121445	F	7	Bilateral iris and chorioretinal coloboma with bilateral microphthalmia	Developmental delay, microdontia		Yes	
	50	SG130194	F	7	Bilateral optic disc coloboma with unilateral microphthalmia		Mother and maternal grand-father with optic disc coloboma and microphthalmia	No	<i>TFAP2A: c.1037_1038del, p.(Lys346fs*84)</i>

F female, M male, R right, L left.

the major functional frizzled domain of the protein (UniProt: Q13467). The loss of the highly conserved amino acid *Histidine* (Suppl.data2) with the insertion of two novel amino acids *Arginine* and *Asparagine* is predicted to be damaging by in silico analyses (Human Splicing Finder, GeneSplicer, ESEfinder). Due to the unavailability of parental DNA, segregation pattern could not be established. According to the ACMG guidelines [16], the variant is considered as likely pathogenic (PM1, PM2, PM4 and PP3). The ClinVar accession number for this variant is SCV000998821.

Patients 3 and 4: familial bilateral isolated coloboma

Patient 3 (SG170145), a 26-year-old woman and her mother (patient 4) are the only two symptomatic members in their family of French origin. The daughter (patient 3) has a bilateral iris and chorioretinal coloboma affecting the macula and the optic nerve, with secondary congenital nystagmus and high myopia (−11 diopters in the right eye, −4.75 diopters in the left eye). Her mother has a bilateral OD coloboma. In both affected members of this family, a heterozygous in-frame deletion of 22 amino acids **c.1181_1246del** [p.(Asn394_Gly415del)] was

identified, reaching the Frizzled domain of *FZD5*. The core functional domain is deprived of 22 highly conserved amino acids (Suppl.data2) and was predicted to be damaging by in silico analyses (Human Splicing Finder, Gene splicer, MaxEnt, EX-SKIP). This variant was never reported in the literature and is absent from gnomAD. According to the ACMG guidelines [16], the variant is considered as likely pathogenic (PM1, PM2, PM4 and PP3). The ClinVar accession number for this variant is SCV000998822.

(Likely) pathogenic variants in *RARB*, *MAB21L2*, *RBP4* and *TFAP2A*

A de novo *RARB* missense variant (NM_000965: c.872A > T, [p.(His291Leu)]) was identified in patient 16 (ADN150083), a 6-year-old boy with bilateral iris coloboma and diaphragmatic hernia. De novo missense variants in *RARB* are known to be deleterious and involved in *RARB*-related PDAC syndrome [17]. This variant was predicted to be damaging by in silico analyses (SIFT, PolyPhen-2, MutationTaster). It was never reported in the literature and is absent from gnomAD. According to the ACMG guidelines [16], the variant is considered as likely pathogenic

Table 2 Clinical and molecular data of the four patients with *FZD5* variants.

Reference number	Sex	Age (years)	Familial history	<i>FZD5</i> variant	Coloboma features		Associated features
					Right eye	Left eye	
Patient 1 ADN120143	F	10	None	c.820del p.(Leu274Cysfs*73)	Iris, choroidal and optic disc coloboma	Choroidal and optic disc	None
Patient 2 ADN140259	F	43	None	c.1081_1082insGAA p.(His361delinsArgAsn)	Optic disc	Iris and optic disc	Right cortical cataract Bilateral congenital deafness Neurocognitive difficulties
Patient 3 SG170145	F	26	Daughter of patient 4	c.1181_1246del p.(Asn394_Gly415del)	Bilateral iris and chorioretinal coloboma affecting the macula and the optic nerve	Bilateral iris and chorioretinal coloboma affecting the macula and the optic nerve	Right posterior cataract Congenital nystagmus and high myopia
Patient 4 ADN190002	F	52	Mother of patient 3	c.1181_1246del p.(Asn394_Gly415del)	Bilateral optic disc	Bilateral optic disc	None

(PS2, PM1, PM2 and PP3). The ClinVar accession number for this variant is SCV000803842.

A novel heterozygous null variant in *MAB21L2* (NM_006439: c.1A > C; p.[Met1?]) was then identified in patient 40 (LGN120039), a 30-year-old women with bilateral colobomatous microphthalmia, intellectual disability, autistic features, craniofacial dysmorphism with scaphocephaly, but without rhizomelic skeletal dysplasia as described in the initial report [18]. Of note, array CGH was normal. Her father was also heterozygous for the c.1A > C, but affected only by isolated bilateral colobomatous microphthalmia. This null variant affecting the initiation codon was never reported in the literature and is absent from gnomAD. According to the ACMG guidelines [16], the variant is considered as likely pathogenic (PVS1 and PM2). The ClinVar accession number for this variant is SCV001364581.

A heterozygous *RBP4* variant (NM_006744: c.569-1G > A, p.?) was identified in patient 27 (SG102327), a 75-year-old men with isolated and sporadic bilateral retinal coloboma. This null variant was never reported in the literature and is absent from gnomAD. Due to the unavailability of parental DNA, segregation pattern could not be established. According to the ACMG guidelines [16], the variant is considered as likely pathogenic (PVS1 and PM2). The ClinVar accession number for this variant is SCV001364582.

Finally, an heterozygous *TFAP2A* variant (NM_003220: c.1037_1038del, [p.(Lys346fs*84)]) was identified in patient 50 (SG130194), a 7-year-old girl presenting with bilateral OD coloboma and unilateral microphthalmia as her mother and maternal grandfather. This null variant, transmitted by the symptomatic mother, is absent from gnomAD and was once reported in the Clinvar database as pathogenic (SCV000747548.1). According to the ACMG guidelines [16], the variant is considered as pathogenic (PVS1 and PM2). The ClinVar accession number for this variant is SCV001364583.

Variant of uncertain significance in *PTCH1*

A heterozygous *PTCH1* variant (NM_000264: c.2500T > G, p.Leu834Val) was identified in patient 23 (SG080744), a 15-year-old girl presenting with isolated and sporadic bilateral ocular coloboma (right iris coloboma and left macular and OD coloboma). It was predicted to be damaging by in silico analyses (SIFT, PolyPhen-2, MutationTaster). This variant was never reported in the literature and is absent from gnomAD. Due to the unavailability of parental DNA, segregation pattern could not be established. According to the ACMG guidelines [16], the variant is considered as being of uncertain significance (PM1, PM2, and PP3). The ClinVar accession number for this variant is SCV001364584.

Discussion

Herein, we tested 50 patients with a phenotype of ocular coloboma on a targeted NGS panel of 119 ocular developmental genes. The ocular coloboma phenotype was either “purely isolated” ($n = 31$) or associated with other eye defects (mainly microphthalmia) ($n = 8$), with systemic anomalies ($n = 8$) or both ($n = 3$). Twenty-two percent (11/50) were familial forms.

Four different (likely) pathogenic variants were identified, respectively, in *RARB*, *MAB21L2*, *TFAP2A*, and *RBP4*, genes repeatedly implicated in MAC phenotypes [6], as well as a variant of uncertain significance in *PTCH1*. Two interesting observations have to be made about the *RARB* and *MAB21L2* variants identified in this work.

Firstly, de novo missense variants in *RARB* are known to be responsible for the PDAC syndrome through a gain-of-function mechanism [6, 17]. However, although our patient’s genotype is classic, his phenotype is less so. Indeed, the absence of eye growth defects in patient 16 (ADN150083) is an infrequent feature that has only been reported once in an individual with a *RARB* variant (c.431G > A, p.Arg144Gln) inherited from his mother whom had herself a colobomatous microphthalmia [19].

Then, concerning *MAB21L2*, the genotype is as unusual as the phenotype. Here, we reported the first heterozygous *MAB21L2* variant affecting the initiation codon (c.1A > C) in a gene where almost only missense variants have been described [6]. Solely one nonsense variant (c.840C > G, p.Tyr280Ter) was described by Patel et al. in *MAB21L2* [20] in a patient with isolated colobomatous microphthalmia. Thus, this report of a second null variant in *MAB21L2* is in correlation with the idea that, unlike missense variants that are located in a mutational hotspot which may have a crucial role both in eye and skeletal development [6], null variants in *MAB21L2* could exert their effects only on ocular development.

Besides the identification of variants in *RARB*, *MAB21L2*, *TFAP2A*, and *RBP4*, as well as *PTCH1*, three novel disease-causing *FZD5* variants were identified in this study.

Of note, the recurrent *ACTG1* variant was not detected in our cohort. This is in full accordance with the low frequency observed from the initial description (two patients in around 400 tested) [11] and a recent study testing 66 familial cases of coloboma [19].

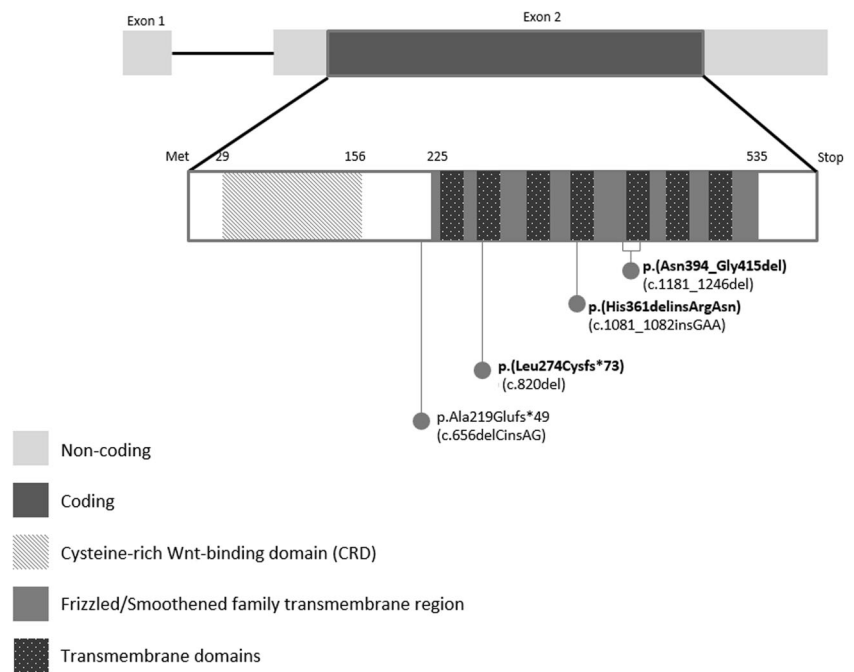
This work allows describing three novel heterozygous *FZD5* likely pathogenic variants considered as disease-causing: one frameshift **c.820del [p.(Leu274Cysfs*73)]**, one in-frame insertion **c.1081_1082insGAA [p.(His361-delinsArgAsn)]** and one in-frame deletion **c.1181_1246del [p.(Asn394_Gly415del)]** in patients with either isolated coloboma or associated with extraocular features.

Surprisingly, our 6% variant detection rate (3/50) is far superior to the previously described, with only one variant identified in 552 patients (172 + 380 patients from two cohorts) with optic fissure defects screened for *FZD5* variants [10].

FZD5 is a single coding exon gene located in the 2q33.3 region, which encodes for the FZD5 protein, a transmembrane receptor for Wnt ligand, composed of an extracellular CRD domain and a transmembrane Frizzled domain (NCBI database). The variant c.656delCinsAG [p.(Ala219-Glufs*49)] previously described and located in the linker region (Fig. 1) results in the production of a truncated protein of 267 amino acids lacking the TMD, with a dominant-negative effect demonstrated by functional analyses [10]. Interestingly, all three novel variants identified herein consist also in small insertions and deletions (Indels) and each of them is affecting one out of the seven TMDs of the frizzled domain (Fig. 1). Because of the closely location of the previously described variant, a dominant-negative effect can be as well a plausible mechanism for the **c.820del** variant [p.(Leu274Cysfs*73)] identified in patient 1, with, possibly, a production of a truncated protein of 346 amino acids. Effects of the two others, the **c.1081_1082insGAA** [p.(His361delinsArgAsn)] and the **c.1181_1246del** [p.(Asn394_Gly415del)], impacting the fourth and the fifth TMD, respectively, have to be determined more precisely, given that a large part of the TMD would be, this time, conserved. At this point, two other mechanisms could also be considered for all these variants: either a loss-of-function effect or nay, a gain-of-function effect, as already suggested in the work of Liu et al. in 2016 [10]. The mechanisms by which the *FZD5* variants lead to coloboma remain elusive. As guidance, no clear genotype–phenotype correlation emerged with the *FZD4* paralogue gene, whose variants lead to autosomal dominant familial exudative vitreoretinopathy [21]. Interestingly, *FZD4* has a highly specific ligand, Norrin, which signaling is lost when swapping *FZD4* linker region (located between the CRD and the Frizzled domain) with the *FZD5* one, suggesting the importance of this region in ligand-specific cellular response [22]. This linker region could also have an importance in *FZD5* function.

Moreover, the gnomAD database contains seven rare truncating variants (with an allele frequency ranging from 1/31,384 to 1/248,778) in the *FZD5* gene, located in the linker region and the Frizzled domain, all respecting the CRD. The occurrence of such variants in an “assumed” asymptomatic population could be explained by either (i) an incomplete penetrance of these variants as illustrated by the original family reported by Liu et al. [10], or (ii) a mild ocular phenotype in those patients (visual impairment ranges from asymptomatic to complete loss of vision, depending on the coloboma size and location).

Fig. 1 Schematic representation of all likely pathogenic variants identified in *FZD5* with protein domains. In bold, the novel variants reported here.



Afterwards, this work allows expanding the phenotypic spectrum of *FZD5* variants, in particular showing the intra/inter-familial variability but also the intra-individual variability such as in patients 1 and 2, who display a different coloboma pattern depending on the eye side. Although the eye phenotype is not detailed for each mutated member of the family described by Liu et al. [10], relevant points regarding the eye phenotype of our mutated patients can be highlighted: (i) the absence of a coloboma affecting solely the iris but rather (ii) the presence in all of a coloboma affecting the optic nerve (like in most of the *PAX2* mutated patients [1]), (iii) the absence of associated M/A and, finally, (iv) the absence of retinal involvement in view of the functional work done by Liu et al. showing the retinal developmental defect when the Wnt-*FZD5* signaling pathway is altered [10]. Moreover, we can notice that patient 2, carrying the **c.1081_1082insGAA [p.(His361delinsArgAsn)]**, displayed a coloboma with extraocular features (bilateral congenital deafness and neurocognitive difficulties). Of note, other defects in Wnt-signaling components are involved in syndromes associating ocular abnormalities, deafness and intellectual deficiency, like in the recessive X-linked Norrie disease [MIM#310600]. Besides, supported by a mice *FZD4*^{-/-} model, Norrin-*FZD4* signaling system has been shown to play a central role in vascular development in the eye and ear, allowing us to assume a link between our patient phenotype and a defect in the Frizzled-protein Wnt-signaling pathway [23]. Unless incidental, it is also interesting to note that *FZD5* is, for the first time, involved in an associated form of coloboma, assuming the first description of a syndromic form. As no

genotype–phenotype correlation seems to emerge (at least for the moment given the low number of reported variants), it is concordant with the high clinical variability observed with most of the genes involved in ocular growth and structural defects. Although occasional, other genes were already described either in syndromic and non-syndromic forms of coloboma, such as *SOX2* or *PAX6* [1, 6].

Thus, the *FZD5* gene is responsible for the coloboma phenotype in 3 out of the 50 families and its implication therefore represents 6% of our cohort, which can be assumed to explain a relatively “high” proportion of cases. Indeed, most of the time, patients with non-syndromic coloboma (>70%) have no identified genetic cause and, despite a wide genetic heterogeneity, each gene explains only a small percentage of cases (less than 3% each) [11]. In our cohort, only 14% of patients (7/50) benefited from a molecular diagnosis. This is in correlation with a recent study that reported a diagnosis rate of 4.5% after screening 38 MAC genes in 66 families affected with syndromic and non-syndromic coloboma [19]. In other words, the chance for identifying a genetic underlying cause is weak in the presence of coloboma. This is well illustrated in the cohort of the 150 MAC patients described by Chassaing et al., in which a genetic etiology was proved only in 7% of the 56 colobomatous microphthalmia cases, against 17% of the 53 non-colobomatous microphthalmia cases and 54% of the 41 anophthalmia cases [9]. Nevertheless, we bring here important pieces of evidence for considering *FZD5* as an important coloboma gene and therefore new insights for the existence of Mendelian genetic bases for the ocular coloboma phenotype.

Facing these latter results, we can also wonder whether microphthalmia, when associated and moderate, is not simply the consequence of a large coloboma. It is classically admitted that structural eye defects of the MAC spectrum represent a phenotypic continuum, and that eye growth defect is a mechanism for coloboma occurrence because fissure margins cannot appose properly. But other mechanisms could be possible, like failure of epithelial fusion. Thus, the coloboma phenotype could be considered as a full clinical entity and the associated microphthalmia as a consequence in the manner of a “sequence”. In addition, the relatively “high” frequency of *FZD5* involvement in coloboma phenotype definitely contrasts with the absence of *FZD5* variants identified to date in anophthalmia and non-colobomatous microphthalmia patients. M/A phenotypes are genetically explained in <50% of the patients [6]. Even novel sequencing technologies, such as WES studies, in cohorts of M/A patients, resulted in a detection rate similar to that obtained by the targeted sequencing of a panel of the main known genes [6].

Advances in genetics have to be made in the field of optic fissure closure, both to improve the diagnosis rate and genetic counseling delivered to the patients and their families. The detection rate by using screening of genes known to be involved in non-syndromic ocular developmental defects remains low with regard to the familial cases (22% in this study). Adding *FZD5* is useful and cost-effective. Some new genes will probably be identified in the future, in association with the generalization of WES studies approaches. However, other molecular non-mendelian mechanisms, such as gene expression regulation or epigenetic processes, could exist and wait to be discovered by using whole genome or methylation studies. Although progress need to be done, *FZD5* can from now be considered as a major gene of ocular coloboma and added in NGS panel dedicated to coloboma diagnostic procedures.

URLS

- *ClinVar: <https://www.ncbi.nlm.nih.gov/clinvar/>
- *ESEfinder: <http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home>
- *ELprep: <https://github.com/ExaScience/elprep>
- *GenBank: <https://www.ncbi.nlm.nih.gov/genbank/>
- *GnomAD: <http://gnomAD.broadinstitute.org>
- *Gene splicer: <https://ccb.jhu.edu/software/genesplicer/>
- *Human Slicing Finder: <http://www.umd.be/HSF>
- *NCBI Database: <https://www.ncbi.nlm.nih.gov/>

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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