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Molecular investigation in Orofacial Clefts with Microphthalmia-Anophthalmia-Coloboma spectrum

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Abstract

Orofacial clefts (OC) are the most common birth defects in humans and approximately 30% of them form the group of syndromic orofacial clefts (SOCs). Microphthalmia/anophthalmia/coloboma spectrum (MAC) can be associated with OC, however the genetic etiologies of OC-MAC have been poorly characterized. This study describes genomic findings among individuals with OC-MAC recorded in the Brazilian Database on Craniofacial Anomalies (BDCA). Chromosomal microarray analysis (CMA) and Whole exome sequencing (WES) were performed in 17 individuals with OC-MAC. Genotype-phenotype correlation was based on clinical data available at the BDCA and on re-examination. No copy number variants (CNVs) classified as likely pathogenic or pathogenic were detected by CMA. WES allowed a conclusive diagnosis in six individuals (35.29%), two of them involving the *CHD7* gene. Variant of uncertain significance (VUS) possibly associated to the phenotypes were found in six other individuals. Among the individuals with VUSes, three individuals presented variants in genes associated to defects of cilia structure and/or function. Investigation by WES seems to be the most effective method for diagnosis in OC-MAC. This study also reinforces the genetic heterogeneity of OC-MAC, highlights the presence of the *CHD7* gene, and the importance of genes related to ciliopathies in this phenotype.

Introduction

Orofacial clefts (OC) are the most common birth defects with an estimated prevalence of 1:700–1:1.000 newborns, with clinical and etiological heterogeneity [1]. Among OC, approximately 30% are syndromic orofacial clefts (SOCs) [2].

Microphthalmia/anophthalmia/coloboma spectrum (MAC) is sometimes described in individuals presenting with OC. According to Schraw et al. [3] these two birth defects co-occur more often than expected than if they were independent events. MAC represents distinct phenotypes from the same eye malformation. The most common etiologies of this spectrum are monogenic conditions and genomic imbalances [4].

The utility of chromosomal microarray analysis (CMA) for diagnosis of SOCs and ocular developmental anomalies (ODA) including MAC has already been demonstrated, with a diagnostic yield for typical SOCs between 9% [5] and 25.3% [6] and 13% for ODA [7]. Whole exome sequencing (WES) on trios has shown that several genes are implicated in MAC, including some that are increasingly expressed in many types of retinal cells [8]. However, the genetic background of the OC-MAC has been poorly characterized in literature so far.

The aim of this study is to describe the molecular investigation in a series of Brazilian cases presenting OC-MAC.

Material And Methods

Cohort

This study used primary data recorded through the Brazilian Database on Craniofacial Anomalies (BDCA) and biological samples from its associated biorepository and by the 10 other BDCA-collaborating hospitals located in the Northeast, Southeast and Southern regions of Brazil [9, 10]. The final cohort was composed of 17 non-related individuals with OC-MAC.

DNA Samples

Genomic DNA samples stored in the BDCA biorepository were obtained from peripheral blood samples using phenol/chloroform extraction, following a standard protocol at the Human Cytogenetics and Cytogenomics Laboratory - School of Medical Sciences – UNICAMP. All samples were purified by Microcon-30kDa Centrifugal Filter Unit with Ultracel-30 membrane (Millipore, Billerica, MA, USA).

Chromosomal Microarray Analysis (CMA)

CMA was performed using the CytoScan™ 750K chip from Affymetrix® (Thermo Fisher Scientific Inc. - Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions for all individuals. Data analysis was performed using the Affymetrix Chromosome Analysis Suite version 4.0 (ChAS - Santa Clara, CA, USA). Interpretation and classification of CNVs and regions of homozygosity followed the recommendations of the American College of Medical Genetics and Genomics (ACMG) [11, 12].

Whole exome sequencing (WES)

WES was performed by Macrogen, Inc.© (Seoul, South Korea) using the Agilent SureSelect^{XT} Human All Exon Kit V6 (Agilent Technologies®, Santa Clara, CA, USA) and the Illumina NovaSeq 6000 Sequencing System (Illumina, Inc©, San Diego, CA, USA) to generate paired-end, 2x150bp reads, and with on-target 140x coverage. The raw data was extracted in Fastq format.

WES Data Analysis

OC-MAC individuals were analysed using standard procedure conducted at the Human Genetics Laboratory of the de Duve Institute in Belgium. Raw data (. fasta files) were aligned to the reference human sequence assembly (GRCh37) using BWA 0.7.15 (Wellcome Trust Sanger Institute) and imported to the bioinformatics computing cluster. Variant detection and calling were performed on aligned sequences (.bam files) using

Picard 1.107 for removal of duplicates and quality value recalibration, and GATK 3.3 Haplotype Caller for variant calling (both from the Broad Institute). The variant (.vcf) files thus generated were imported into a database and further analysed using an in-house NGS data-analysis framework called Highlander 14.10.3 (https://sites.uclouvain.be/highlander/), used for variant annotation, filtering, and visualization. Filtering was retained for variants that satisfied the following criteria: (i) pass GATK standard quality-control filters, (ii) within a list of 459 candidate genes for oral clefts; (iii) missense, nonsense, frameshift and splice-site changes; (iv) < 1% allele frequency in the ExAC database of WES from 60,706 unrelated individuals (http://exac.broadinstitute.org/); (v) not detected in samples from individuals with unrelated pathologies (or unaffected controls) in the in-house database of 1800 WES (vi) for missense variants, predicted to affect protein function by at least 3 out of 6 prediction tools (DAMAGING in Sift, DELETERIOUS in LRT, HIGH or MEDIUM in Mutation Assessor, DAMAGING in FATHMM, DISEASE CAUSING (AUTOMATIC or not) in Mutation Taster, a score > 0.5 in Polyphen2 (hdiv or hvar)). After selection of the variants by Highlander (https://sites.uclouvain.be/highlander/) a preliminary classification was performed using what is known about protein function, alignment, constraint scores of the protein (pLI, z-score for missense and synonymous mutations), frequency in databases such as LOVD, Clinvar, and predictions by Varsome (https://varsome.com).

A second analysis of all cases was performed using Varstation [13] as this platform also makes use of the ABraOM database, which contains genomic variants obtained with WES and whole genome sequencing (WGS) from the Brazilian population [14]. In addition, an automatic variant pre-classification was performed, according to the recommendations and guidelines of the ACMG, Association of Molecular Pathology (AMP) and the College of American Pathologists (CAP) [15]. Each variant pre-classification was verified by the analyst and reclassified when necessary.

After these procedures, all cases were clinically reviewed, using data from the BDCA and during a consultation, when possible. The variants were assigned as causative of the phenotype, possibly related to, and non-causative based on case-by-case genotype-phenotype correlation, after revision of individual medical records, public databases with reports of the relationships among human variations and phenotypes (ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and DECIPHER (https://www.deciphergenomics.org/), information related to animal models and the scientific literature.

Variant of uncertain significance (VUS) in genes in which a known phenotype was similar to the one of the individuals were considered causative, as suggested by Johnson et al. [16].

Validation by Sanger sequencing

All variants were confirmed by Sanger sequencing. The Ensembl Genome Browser (assembly GRCh37/hg19 and GRCh38/hg38) was used to design the primers. The fragments of interest were amplified by Polymerase Chain Reaction and purified using Exo-Sap enzyme (Applied Biosystems ™). Sequencing reaction was performed with BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems ™). Sequence reading was performed through automated capillary electrophoresis (ABI 3500xL Genetic Analyzer, Applied Biosystems ™) and the results were analyzed using the CodonCode Aligner© software (CodonCode Corporation, Dedham, MA, USA). Co-segregation analyses were performed when parents were available.

Results

We evaluated 17 unrelated individuals, 4 male, 13 female. Ages varied from 0 to 30 years. No CNVs classified as likely pathogenic or pathogenic were detected by CMA. Variants identified by WES were considered causative in six individuals and possibly associated to the phenotype in six. Variants in the *CHD7* gene were considered causative in two cases (patients 1 and 2) and probably contributing to the phenotype in another patient (patient 11). Other causative variants were found in the following genes: *PTPN11*, *TP63*, *TFAP2A*, and *POMT1*. In five cases, no variants classified as VUS, likely pathogenic or pathogenic were detected by WES (patients 13, 14, 15, 16 and 17). Detailed clinical data and WES results are depicted in Table 1.

Table 1
Clinical features and WES results of individuals with OC-MAC

ID	Gender	OC	MAC	Other clinical data	WES	ACMG classification	Diagnostic Status
1	F	Bilateral CLP	Optic disc coloboma, microphthalmia	Microcephaly, abnormal ear morphology, micrognathia; short neck, hypoplastic nipples, ventricular septal defect hypoplastic labia minora and labia majora; neurodevelopmental delay and intellectual disability	*CHD7*:c.4603_4604dup:p.(Lys1536fs) Het. *de novo*	Pathogenic	**Conclusive** CHARGE S., AD
2	F	CLP	Synophris; right anophthalmia, left eye microphthalmia	Genu valgus	*CHD7*:c.5420A > C:p.(Asn1807Thr) Het. / NP DNA	Likely Pathogenic	**Conclusive** *CHD7* spectrum, AD
3	F	Left CLP	Left anophthalmia	Short stature, café-au-lait spots, multiple lentigines joint hyperflexibility, mitral prolapse and mild mitral insufficiency	*PTPN11*:c.1529A > G:p.(Gln510Arg) Het. / NP DNA	Likely Pathogenic	**Conclusive** Noonan with Multiple Lentigines S., AD
4	F	Left CLP	Left microphthalmia, right eye cataract	Abnormal nasolacrimal system morphology, tricuspid regurgitation, limb asymmetry, 5th finger clinodactyly and mild intellectual disability	*TP63*:c.1813C > T:p.(Arg605Trp) Het. / NP DNA	Likely Pathogenic	**Conclusive** ADULT S., AD
5	F	Bilateral CLP	Left eye retinal coloboma	Abnormality of hair texture, low-set ears, narrow external auditory meatus, branchial anomaly, supernumerary nipple, atrial septal dilation (aneurism) and kidney cyst	*TFAP2A*:c.874G > A:p.(Glu296Lys) Het. / NP DNA	Pathogenic	**Conclusive** Branchiooculofacial S., AD
6	F	Left CLP	Left eye microphthalmia and cataract, right eye anophthalmia	Microcephaly, encephalocele, eyelid ptosis, left eye, micrognathia, retrognathia and neurodevelopmental delay	*POMT1*:c.987-3C > G Hom. / NP DNA	VUS	**Conclusive** *POMT1* clinical spectrum, AR
7	F	Bilateral CLP	Retinal detachmen; and right eye microphthalmia	Neurodevelopmental delay, hydrocephaly, large fontanelles, low-set ears, wide nasal bridge and inguinal hernia	*PORCN*:c.468G > T:p.(Met156Ile) Het. / NP DNA	VUS	**Possible association** Isolated microphthalmia, XL

ID	Gender	OC	MAC	Other clinical data	WES	ACMG classification	Diagnostic Status
8	F	CLP; absent uvula	Left anophthalmia; right eye coloboma and cataract, abnormal eyebrow morphology; nystagmus; ectropion strabismus	Microcephaly, patchy alopecia, high anterior hair line, abnormality of hair texture, abnormality of the scalp, microtia, protruding ear, low-set ears, abnormality of the pinna, micrognathia, retrognathia, abnormality of dental structure, hypoplasia of the nipples, hemiatrophy, clinodactyly of the 5th finger, cutaneous syndactyly of the toes, aplasia/hypoplasia of the nails and hypopigmented macules	*BMP4*:c.171C > G:p.(Phe57Leu) Het. / NP DNA	VUS	**Possible association** Microphthalmia, Syndromic 6, AD
9	M	Bilateral CL	Blepharophimosis and ankyloblepharon; right eye anophthalmia; left eye microphthalmia	Skull asymmetry, plagiocephaly, microcephaly, posteriorly rotated ears, low-set ears, deep plantar creases, atrial septal defect, ventricular septal defect, patent foramen ovale, patent ductus arteriosus and severe neurodevelopmental delay. First degree cousin with congenital cardiopathy	*GDF1*:c.928G > C:p.(Gly310Arg) Het. / NP DNA	VUS	**Possible association**
					GRHL3:c.580C > T:p.(Pro194Ser) Het. / NP DNA	VUS	**Possible association**
10	F	Unilateral CLP	Bilateral blepharophimosis and microphthalmia	Large fontanelles, macrocephaly, hirsutism, preauricular skin tags, diastasis recti, deep plantar crease, atrial septal defect, ventricular septal defect and truncus arteriosus	*DYNC2H1*:c.7858C > T:p.(Arg2620ter) Het. / NP DNA	Likely Pathogenic	**Possible association**
					KIAA0586:c.3G > A:p.(Met1?) Het. / NP DNA	VUS	**Possible association**
					WDR34:c.991G > A:p.(Gly331Arg) Het. / NP DNA	VUS	**Possible association**
11	F	Atypical orofacial cleft (a facial cleft	Right anophthalmia	Patent ductus arteriosus	*LMNA*:c.1930C > T:p.(Arg644Cys) Het. / NP DNA	VUS	**Possible association**

ID	Gender	OC	MAC	Other clinical data	WES	ACMG classification	Diagnostic Status
		from the right upper lip to the right eye)			*INTU*:c.2512T > C:p.(Cys838Arg) Het. / NP DNA	VUS	**Possible association**
					SOX1:c.401A > G:p.(Lys134Arg) Het. / NP DNA	VUS	**Possible association**
					CHD7:c.8957G > C:p.(Gly2986Ala) Het. / NP DNA	VUS	**Possible association**
12	F	Unilateral CLP	Right eye microphhalmia	No other findings	*KIF7*:c.3248dupA:p.(Asn1083fs) Het. / NP DNA	Likely Pathogenic	**Possible association**
					RPGRIP1L:c.3745G > T:p.(Asp1249Tyr) Het. / NP DNA	VUS	**Possible association**
					RPGRIP1L:c.1165A > G:p.(Ile389Val) Het. / NP DNA	VUS	**Possible association**
					FLNB:c.107G > A:p.(Arg36His) Het. / NP DNA	VUS	**Possible association**
					ARHGAP29:c.2120G > A:p.(Arg707His) Het. / NP DNA	VUS	**Possible association**
13	M	CP; absent uvula	Left eye microphthalmia	Microcephaly, hydrocephaly, hypertelorism, eyelid ptosis, epicanthus, wide nasal bridge, inguinal hernia and neurodevelopmental delay	NA		
14	M	Left alveolar cleft	Right eye microphthalmia; left optic disc coloboma	Phimosis	NA		
15	F	Bilateral CLP	Synophris; bilateral iris coloboma	Microcephaly, abnormality of hair pigmentation and atrial sept defect	NA		
16	F	Atypical orofacial cleft	Right anophthalmia, left eye microphthalmia with iris and choroid coloboma	Hydrocephaly, agenesis of corpus callosum, severe intelectual disability and consanguinity (parents are first degree cousins)	NA		

ID	Gender	OC	MAC	Other clinical data	WES	ACMG classification	Diagnostic Status
17	M	CP	Microphthalmia	Macrocephaly, brachycephaly, abnormal hair pattern, facial asymmetry, micrognathia, short neck, pectus excavatum, malar hypoplasia, hypoplastic nipples and neurodevelopmental delay	NA		

Abbreviations: ID: identification; OC: orofacial cleft; Het: heterozygosis; Hom: homozygosis; AD: autosomal dominant; AR: autosomal recessive; XL: X-linked; S. syndrome; CLP: cleft lip and palate; CP: cleft palate; CL: cleft lip; NA: no alterations; NP DNA: no parental DNA. hg38: patients 11 and 12; hg19: all other patients.

Discussion

Many structural anomalies have been described in association with OC, including the MAC spectrum [17]. The presence of CP, CL with or without CP (CL+/-P) seems to represent a risk of concomitant microphthalmia and/or anophthalmia [18]. Few case series studies have focused on the co-occurrence of MAC spectrum and other birth defects. Studying a cohort of 415 live births with microphthalmia/anophthalmia/coloboma, Roos et al. [19] reported that 13% presented with coloboma and OC, and 25% with microphthalmia or anophthalmia and OC.

Our study presents the investigation of genetic variants using CMA and WES, in individuals with OC-MAC in which clinical assessment was performed by experienced dysmorphologists, following the same protocol of data collection and record [9].

OC and MAC are phenotypically heterogeneous. Throughout the years, over 300 genes have been implicated in the etiology of orofacial clefts, including the *IRF6* gene, which accounts for 2% of cases of SOCs, represented by Van der Woude syndrome (OMIM #119300) and is also responsible for many cases of non-syndromic clefts (NSOCs) [20]. The genes *GRHL3*, *TBX1*, *TP63*, and *LRP6* are also important genes related to SOCs that are also described in NSOCs [21, 22]. In addition, more than 90 genes related to MAC spectrum have been identified so far, most of them transcription factor genes (*SOX2*, *OTX2*, *VSX2* and *PAX6*), and the retinoic acid pathway genes (*STRA6*, *RARβ*, and *ALDH1A3*) [23]. However, genomic imbalances and sequence variants in these genes were not detected in our cohort.

Since chromosomal imbalances are seen in about 15–20% of patients with intellectual disability or multiple congenital defects [24] and are found in up to 44.4% of individuals with microphthalmia and/or anophthalmia [19], searching for genomic imbalances was the first strategy chosen for diagnosis. However, despite previous studies have demonstrated the importance of CMA in the investigation of syndromic MAC spectrum [4] and SOCs [5], none of the patients in this OC-MAC cohort presented pathogenic genomic imbalances, hence why the study was pursued with WES analysis.

Genotype-phenotype correlation

In the present study, causative variants in the *CHD7* gene were detected in 2/17 cases. Patients 1 and 2 fulfilled the diagnostic criteria for the *CHD7* phenotypic spectrum expansion [25]. Variants in the *CHD7* gene were also detected in two individuals from a cohort of 67 trios with MAC investigated by WES [8] and this seems to be an important gene in the OC-MAC etiology.

After genotype-phenotype correlation, etiological heterogeneity among individuals of this cohort included a causative variant in exon 13 of the *PTPN11* gene in patient 3, that is responsible for Noonan Syndrome with Multiple Lentigines (OMIM #163950) [26], and a causative variant in *TFAP2A* gene, leading to Branchiooculofacial syndrome (BOFS - OMIM #113620) (Patient 5). Patients 4 and 6 have causative variants involving genes *TP63* and *POMT1*, respectively, with some particularities that are mentioned below.

Patient 4 met the criteria for ADULT syndrome (OMIM #103285), which is one of syndromes included in the wide clinical spectrum of *TP63* [27]. The association of *TP63* variants and microphthalmia or cataract, as presented in our patient, has not been described before. However, reports of two patients with glaucoma associated to anterior segment dysgenesis and *TP63* variants suggest that this gene might participate in the anterior eye segment embryogenesis [28]. Therefore, it seems that the eye anomalies detected in our patient could be explained by the

TP63:c.1813C > T:p.(Arg605Trp) variant and should be considered a phenotype expansion of the *TP63* gene. Further functional studies and clinical examples would need to be performed and identified to verify this claim.

Despite the VUS classification following the ACMG guidelines, some individuals who had VUSes presented with a phenotype strongly indicative of the disorders associated to the mutated gene. Further functional studies could also help to identify if these variants contribute to the diagnosis.

Patient 6 presented a splice site variant in the *POMT1* gene (c.987-3C > G), classified as VUS. The phenotype included microcephaly, encephalocele, microphthalmia, anophthalmia, cataract, CLP and neurodevelopmental delay. These features are considered part of the clinical phenotypic spectrum of *POMT1* gene and would allow a diagnostic conclusion [29]. Therefore, this variant was considered causative, as suggested by Johnson et al. [16].

In addition, there are some variants that could not be considered the exclusive cause of the respective patient's phenotypes – including some heterozygous variants detected in genes with an autosomal recessive mode of inheritance. However, they seem to contribute to a specific malformation observed: microphthalmia in patient 7, anophthalmia, OC and digital anomalies in patient 8, and congenital cardiac malformations and embryonic anterior axis patterning in patient 9.

Patient 7 presented a heterozygous variant in the *PORCN* gene that could be related with his MAC phenotype, based on a report of a family with male individuals with variants in *PORCN* and presenting with isolated microphthalmia [30].

In patient 8, the *BMP4* variant could be related to at least part of her phenotype, including low-set ears, micrognathia, retrognathia, anophtalmia, CLP, clinodactyly and syndactyly (OMIM #607932).

In Patient 9 we found variants in *GDF1* and *GRHL3* genes. Variants in *GDF1* cause autosomal dominant multiple cardiac congenital defects [31]. Hence, in this patient, this variant could be related with the cardiac congenital malformations observed. The *GRHL3* gene was described as a cause of Van der Woude syndrome (OMIM #606713) and NSOCs, including in the Brazilian population [32]. Clinical follow-up, additional case reports and functional studies would help for a better understanding of these variant's contributions to the phenotype.

VUSes in other genes associated with oral clefts are present in patients 11 (*SOX1*) and 12 (*FLNB* and *ARHGAP29*) [33]. Other VUSes, which possibly have an additive effect in a polygenic mode of inheritance, will be discussed next (patients 10, 11 and 12).

The interpretation and reclassification of VUSes have been increasingly discussed in literature. In general, the guidelines of the ACMG and AMP consider the phenotype as supporting criteria for variant classification if it is highly specific for a unique syndrome or disease [15]. However, many rare variants detected in individuals with well-known syndromes and highly specific for them were classified as VUS according to the current classification criteria [34] and there has been a growing tendency to consider the phenotype as more important evidence for variant classification [16]. This is particularly true when considering craniofacial anomalies, which comprise a group of conditions with etiological and phenotypic heterogeneity.

Ciliopathy genes in OC-MAC

Some variants detected in patients 10, 11 and 12 are in genes that encode primary cilia structures (*DYNC2H1*, *KIAA0586*, *WDR34*, *INTU*, *RPGRIP1L* and *KIF7*) [35–40], and many of these genes have already been described as causative or contributing to phenotypes which include OC and MAC (*KIAA0586*, *INTU*, *RPGRIP1L*, *KIF7* and *LMNA*) [36, 38, 39, 41–43]. However, possible mechanisms involved in these complex birth defects have not been pointed out.

Patient 10 presented a likely pathogenic heterozygous variant in *DYNC2H1* gene, and VUSes in *KIAA0586* and *WDR34* genes. The *DYNC2H1* gene encodes an intraflagellar transport (IFT) protein of cilia [35]. The *KIAA0586* gene is the third most frequent mutated gene detected to cause Joubert syndrome with coloboma (OMIM #616490) [36]. The *WDR34* gene is described as the cause of Short-Rib Thoracic Dysplasia 11, with or without polydactyly (OMIM #615633), an autosomal recessive skeletal ciliopathy.

The *INTU* gene coordinates ciliogenesis in vertebrates and was recently described as having a role in Orofaciodigital syndrome XVII (OMIM #617926) and Short-Rib Thoracic Dysplasia with polydactyly 20 (OMIM #617925) in a few patients [38]. There is a description of one individual, with compound heterozygous *INTU* variants, with microphthalmia and median CLP among other malformations [38]. This variant was found in patient 11, who also has VUSes in *CHD7*, *LMNA*, and *SOX1*.

In patients 10 and 11, compound heterozygosity was excluded based on analysis of other variants or CNVs in this group of genes.

In patient 12, who has CLP and microphthalmia, two variants in *cis* in *RPGRIP1L* were detected, both classified as VUS.

The *RPGRIP1L* gene encodes a protein that localizes to the central body and centrosomal structures of primary cilia and the inactivation of its ortholog in murine model leads to a phenotype like Meckel syndrome (MKS – OMIM#611561) and Cerebello-oculo-renal syndrome (Joubert syndrome type B – OMIM #608091) [39]. It is also implicated in COACH syndrome 3 (OMIM #619113), which can include coloboma among phenotypic findings [41]. Three fetuses from two unrelated families with MKS and biallelic variants in *RPGRIP1L* were described with median CP, microphthalmia, and other ocular malformations, among other major anomalies [39].

Patient 12 also has a likely pathogenic variant in *KIF7* gene. This gene participates in *SHH* regulation, encodes a motor protein of primary cilia [40], and is involved in NSOCs [44]. In addition, CP was also described in individuals with variants in *KIF7* [45].

Disruption of primary cilia structure or their function could be important causes of OC-MAC, at least, they represent part of the mechanisms that leads to them. The hypothesis that OC-MAC phenotype is the result of a disruption in ciliopathy genes has already been discussed in literature, and demonstrated in animal models [46] and in humans [47]. Clinical and molecular findings in the three patients herein described reinforce this possibility. However, future functional studies are still needed to confirm this hypothesis.

In addition, CL+/-P and microphthalmia are both considered midline defects, since they can be associated with a disruption in anterior-posterior or left-right axis patterning [48]. It is noteworthy that, in the present cohort, cardiovascular defects were present in eight individuals out of 17 (47.06%) and, in general, the heart is the most commonly affected organ during laterality defects [48].

Digenic/oligogenic mode of inheritance

The genes *DYNC2H1, LMNA*, *KIF7* and *INTU* have already been reported in patients with conditions that present a digenic mode of inheritance [38, 42, 43, 49].

The *DYNC2H1* gene is involved in a digenic mode of inheritance in a report of a patient with Short-Rib Polydactyly Syndrome (SRPS – OMIM #613091) with a heterozygous variant in *NEK1* gene, resulting in a premature stop codon, and a missense variant affecting a splice site in *DYNC2H1* gene [49].

Digenic inheritance is also one of the most accepted explanations for the diversity of phenotypes and the variable penetrance of the *LMNA*:c.1930C > T:p.(Arg644Cys) variant [42, 50]. The *LMNA*:c.1930C > T:p.(Arg644Cys) variant seen in patient 11, who presents with anophtalmia, atypical OC, and congenital heart disease is related to many phenotypes with diverse presentations and variable penetrance [42]. Although the clinical picture is not very similar, there is a report of an individual with atypical findings of laminopathies, including microphthalmia and cataract, who inherited this variant from the unaffected mother [42].

The *KIF7* gene has been described in ciliopathies either as a modifier of *GLI3* and *NPH1* genes [40, 45] or as having a digenic inheritance pattern with *KIAA0556* gene in an individual with coloboma and CLP among other major anomalies [43]. Putoux et al. [45] carried out functional studies with morpholino and showed evidence that hypomorphic *KIF7* alleles interacts in *trans* with Bardet Biedl syndrome loci (OMIM #209900) [45], there-fore showing the potential for *KIF7* to interact with other ciliary genes, exacerbating their phenotypes.

Concerning *INTU*, Toriyama et al. [38] described a patient with clinical findings suggestive of SRPS probably caused by digenic inheritance, with the participation of one heterozygous variant of this gene [38].

From these results and the information found in literature, including case reports and functional studies, it is possible to consider that the variants detected in individuals 10, 11 and 12 could contribute with the phenotypes observed by means of digenic or polygenic inheritance and, maybe, also as genetic modifiers of other genes.

Role of WES for diagnosis of OC-MAC

WES detected causative variants in six individuals (35.29%) with OC-MAC in this cohort. Currently many variants are still classified in the literature and databases as VUS due to shortfall of reports on such rare phenotypes. The underrepresentation of the Brazilian population in databases is another possible bias for the interpretation of these variants. Considering the aspects mentioned above, the genotype-phenotype correlation could be inferred in another six individuals (35.29%) based on gene function, minor allele frequency in population databases and protein deleterious effects demonstrated by in silico prediction studies (detailed description and in silico prediction are available in supplementary material).

Concluding Remarks

No genomic imbalances were observed, and WES detected causative variants in 35.29% (6/17) of the patients; two of them involving the *CHD7* gene. These results suggest that WES should be complimentary to CMA, even though here, in these 17 individuals, clearly it was most effective approach in the molecular investigation of OC-MAC cases. In general, the spectrum of *CHD7* should be considered in the clinical evaluation of OC-MAC.

In three patients with OC-MAC, results suggest the possibility that their phenotypes are related to defects of structure and (or) function of cilia, highlighting the importance of the pathways involved in cilia in the etiology of OC-MAC. However, further functional studies are needed to corroborate this hypothesis.

Many VUS detected in our cohort could be contributing to the phenotypes investigated and we hope that the results presented here can aggregate information on the etiology of SOCs, facilitating the reclassification of these variants in the future.

Declarations

Data Availability Statement

The data generated or analyzed during this study are available from the corresponding author upon reasonable request.

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Conflict of Interest:

The authors have no conflict of interest to declare.

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Author contributions

MAT performed clinical evaluation, genotype-phenotype correlation and wrote the manuscript; MMC performed laboratory tests, CMA and WES data analyses through Varstation and revised the manuscript; EP performed the WES data analyses through Highlander and variant evaluation and revised the manuscript; ILM collaborated with the design of the study and revised the manuscript; EMR performed clinical evaluation and revised the manuscript; ELM performed clinical evaluation and revised the manuscript; RH developed the data analysis program used for WES analysis; TAPV collaborated with the design of the study, CMA analysis and revised the manuscript; MV supervised the WES studies, provided funding and revised the manuscript; VLGSL designed the study, performed clinical evaluation, provided funding, performed genotype-phenotype correlation and revised the manuscript.

Ethical Approval

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of University of Campinas (protocol numbers: 35316314.9.1001.5404 and 85020018.8.0000.5404). Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

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