UNIVERSITA' DEGLI STUDI DI PAVIA

Dipartimento di Medicina Molecolare

Whole Exome Sequencing in Hereditary Ocular Disease Leads to Recognition of Novel and Recurrent Disease-Causing Mutations: Pros and Cons

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Dottorato di Ricerca in

Genetica, Biologia Molecolare e Cellulare

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Abstract

Background: Hereditary ocular diseases show an extreme genetic and phenotypic heterogeneity, ranging from mild retinal dysfunctions to severe congenital forms of blindness. Most of the many genes associated with these diseases encode signaling and structural proteins are involved in the developing eye. Another category of serious visual impairments concerns the degeneration of neurons as photoreptors, related to the various forms of retinitis pigmentosa. All modes of Mendelian inheritance occur and many are sporadic cases. Identification of the underlying genetic basis for families and affected individuals, understanding genotype-phenotype correlations and developing therapeutic approaches are therefore highly challenging.

Methodology: In this study whole exome sequencing (WES) was performed on Hiseq2500 platform (Illumina) as a powerful strategy for assessment of multiple candidate disease genes (> 450) at the same time in 11 unrelated families (16 patients) with different eye defects, including microcornea/ coloboma / microphthalmia / anophthalmia, Axenfeld-Rieger syndrome, Retinitis Pigmentosa, Leber congenital amaurosis and Stargardt macular degeneration. Afterward, Sanger sequencing was performed to confirm and determine whether any of the candidate variants co-segregated with the disease phenotype in the families. We evaluated the diagnostic yield, the spectrum of clinical referrals, the challenge of variants' interpretation and the genetic heterogeneity of such diseases.

Results: Our data indicate that this approach enables us to genetically diagnose approximately 80 % of the patients (n = 13) with variant(s) in known disease-associated genes. We revealed four pathogenic variants in *RAB3GAP1* (p.(Tyr958*), *CHD7* (p.Ala1347Glnfs*25), *KCNK9* (p.Gly266Arg), CDH23 (p.Leu1343Phe) and *TULP1* (p.Gly266Val, four of them are novel and not reported in the literature or dbSNP: *RAB3GAP1, CHD7, CDH23,* and *TULP1* . In addition, we identified 6 known disease-associated variants, previously reported, in *USH2A* (p.Asp347Gly

& p.Leu3606Pro), *FOXC1* (p.Asp261Argfs*45), *STRA6* (p.Arg655His), *GUCY2D* (p.Pro130Leufs*36), and *ABCA4* (p.Ser1696Asn). The identified mutation spectrum involved novel variants and previously described recurrent mutations Further, we pointed out two variants probably explaining the abnormal ocular phenotype in: *TULP1* (c.823-17G>C) and *CDH23* (p.Cys1045Phe). The vast majority of mutations have not been reported in the Italian population (only *USH2A*; p.Asp347Gly) .We also identified a novel phenotype for mutations in *KCNK9.* In one family, identified phenotypes were different from the previously reported clinical findings associated with the causative gene in *STRA6.*

Discussion & Conclusions: WES can rapidly identify variants in various families affected by different forms of hereditary ocular diseases in contrast to Sanger sequencing of candidate genes. In fact the genetic and phenotypic heterogeneity of eye diseases impair straightforward genotype-phenotype relationships . Without WES, we would not have been able to arrive at a molecular diagnosis in 13 cases, even by using commercial panels of genes involved in eye defects that, although continuously upadated, do not included all the possible causative genes.We discussed the challenges experienced in data analysis and showed examples of cases where the detection of the causative variant required further investigation due to the low coverage of some genes, or would be valued unlikely applying the standard WES analysis. For instance, in two families, we were not able to detect mutations in genes currently associated with inherited eye diseases in humans or in animal models. These cases are being additionally examined for identification of novel disease-associated gene mutations. Our results support the high heterogeneity among patients with eye malformations and show that the WES can be an expeditious approach to the identification of disease-causing variants in these conditions. This study , expands the clinical and the allelic spectrum of genes associated with eye defect in the Italian population.

Keywords: Hereditary ocular disease, novel pathogenic variants, WES

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Abbreviations

Anterior Segment Dysgenesis	(ASD)
Array - Comparative Genomic Hybridization	(Array-CGH)
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Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources	(DECIPHER)
Exome Aggregation Consortium	(ExAC)
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Human Gene Mutation Database	(HGMD)
Human genome variation society	(HGVS)
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Inherited retinal degeneration	(IRD)
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Stargardt Macular Degeneration	(STGD)
Whole Exome Sequencing	(WES)
Whole Genome Sequencing	(WGS)
World health organization	(WHO)

1 Introduction

On a global scale, 285 million people are estimated to be visually impaired: 39 million are blind and 246 have low vision but 80% of all visual impairment can be prevented or cured. (According to WHO, 2016). Congenital eye defects occur relatively frequently in the human population, despite the fact that they are classified as rare diseases, they affect may be more than two million people worldwide.

These disorders can be distinguished clinically, the disease development is difficult to expect and may vary even within the same family, and can be inherited as autosomal recessive (AR), autosomal dominant (AD), as well as X-linked (XL), mitochondrial traits, or digenic inheritance in some cases. Nevertheless, the majority of cases are sporadic (Berger et al. 2010; Glöckle et al. 2014; Chiang JP & Trzupek K.,2015).

The human eye is a structurally complex system (**Figure 1-1**). DNA variants affecting genes involved in the development of the eye may result in partial or complete blindness since birth, with or without abnormal eye conformation (anophthalmia, microphthalmia,etc), or in severe vision impairement, starting at different ages, due to the specific degeneration of some of the eye components, including the cornea, lens, iris, retina and optic fissure or an abnormal function. Subsequently, partial or complete vision loss is experienced by affected individuals. These diseases are heterogeneous, not only in terms of age of onset, progression and severity of the disease, but correspondingly in terms of their underlying genetics (<https://sph.uth.edu/retnet/> - last accessed 30 October) 2016. Progress in genetic diagnostic capability in patients with developmental eye diseases, including ocular developmental disorders such as, cataract/microcornea, disorders of the anterior segment, anophthalmia /microphthalmia / coloboma also Inherited retinal diseases (IRDs).). In fact, if we exclude tumors, few other

diseases have so far had a translational impact as important as degenerative diseases of the vision. However, in spite of the several disease-genes already identified in this field, the overlap of the clinical features associated with variants in many different underlying genes (genetic heterogeneity) makes hard the molecular diagnosis in most of the retinal degeneration cases. In contrast, in cases of eye developmental defects, the genetic diagnosis is aided by the presence of distinct features and by a less extensive genetic heterogeneity.

Understanding of the genetic basis of an affected patients with Developmental eye diseases represents the first step to a better understanding of the physiological role of the underlying protein and disease pathways, which in turn serves as a starting point for developing therapeutic intervention trials, and since it represents a unique area of research with a significant translational impact which, serves in controlling the progress and in applying gene therapy. Nevertheless, a number of disease genes have been identified, proceedings of a molecular diagnosis are often not clear-cut as there is an overlap in the clinical features associated with mutations in the various underlying disease genes. In some cases, genetic diagnosis may be aided by syndrome identification or a chromosomal anomaly may be revealed. In isolated cases, which are the majority, molecular diagnosis is challenging, as the required sequencing for identification of underlying mutation/s is rarely completed, owing to the lack of an available panel covering all the known genes (Ran et al.2014 &Okazaki et al.2016. It is quite likely that the accumulation of precise genotype-phenotype relationships allows in a short time the identification of a panel of genes whose sequencing identifies the molecular cause in almost all cases and this is the aim of the study presented here.

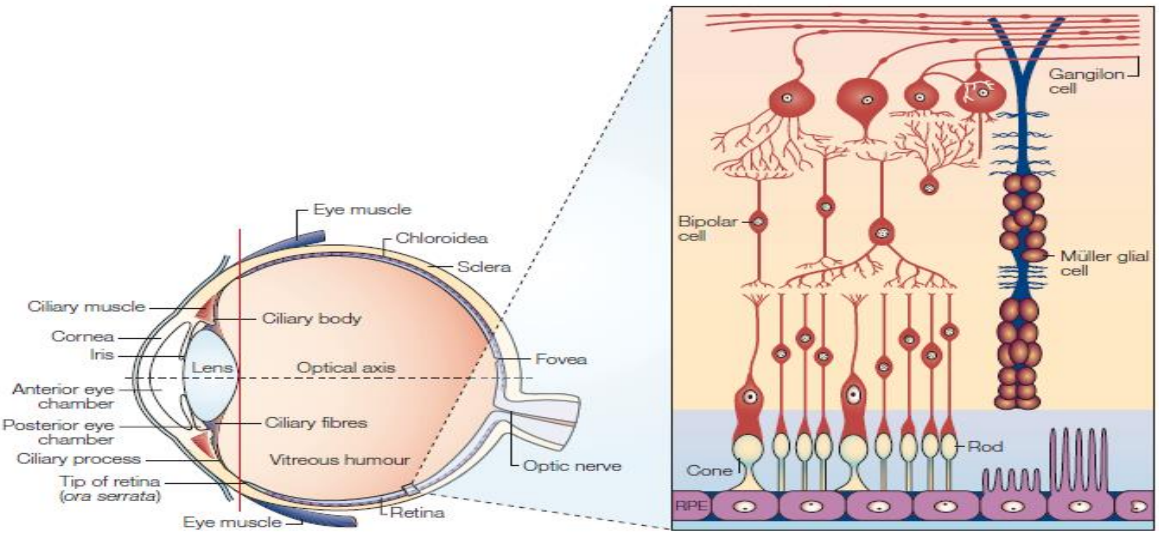

Figure 1-1: Schematic section through an adult human eye

This illustration shows the main tissues of the human eye; note that the optical axis does not coincide with the exit of the optic nerve or with the fovea, which is the area of the highest resolution at the retina. The vertical line divides the anterior segment of the eye (the CORNEA, LENS, IRIS and CILIARY BODY) from the posterior segment (consisting mainly of the VITREOUS HUMOUR, RETINA and the CHOROIDEA). Light enters the eye through the cornea, the anterior chamber and the lens. Before it meets the retina, light has to pass through the vitreous humour. The panel on the right shows a close-up view of the components of the retina, which are (from the outside to the inside of the retina): the retinal pigmented epithelium (RPE), photoreceptor cells (rod and cone), Müller GLIAL CELLS, bipolar cells and GANGLION cells. (Schematic section and legend Adapted from Graw J., 2003.

1.1 Ocular developmental disorders

The development of ocular structures is an extraordinarily controlled and highly conserved process; disruption in this process can result in a variety of different clinical phenotypes (Heavner W&Pevny L., 2012).In the following section I discuss the major phenotypes that I focused on my studies during my dissertation that include microphthalmia, coloboma and

anterior segment dysgenesis which are associated with non-syndromic or syndromic(in some cases-extremely-complex).

1.1.1 Microphthalmia/Anophthalmia/Coloboma Spectrum

Microphthalmia (small eyes; MIM 601186), Anophthalmia (absent eyes; MIM 206900), and coloboma (defective closure of the optic fissure, may well affect the iris, choroid, retina and/or optic disc, typically presenting as absent tissue or a gap; MIM 120200) comprise the MAC spectrum of congenital ocular malformations and consider as birth defects with significantly reduced vision in approximately 1 in 10,000 individuals.

Mutations in several genes, including: *SOX2, ALDH1A3, FOXE3, BCOR, CHD7, STRA6, OTX2, RAX, BMP4, VSX2, GDF6, SIX6, PAX6, VAX1* and *SMOC1* and others can cause MAC spectrum, but there is substantial genetic heterogeneity and >50% of affected individuals do not receive a molecular genetic diagnosis for their birth defect after analysis of the current known causative genes (Choi et al. 2014). MAC can occur as an isolated malformation or can be combined with other ocular abnormalities affecting anterior or posterior segments of the eye (complex MAC) such as Microcornea (is a congenital condition in which the corneal diameter is <10 mm or <9 mm in newborns) or can be a syndrome that affects other organs and tissues in the body, one-third and one-half of affected individuals have microphthalmia as part of a syndrome.

1.1.2 Anterior Segment Dysgenesis (ASD)

ASD or Axenfeld-Rieger syndrome (abnormalities of the front part of the eye; MIM 180500/602482), is important to identify because it is frequently associated with cataracts and increased ocular pressure and developmental glaucoma in about half of affected individuals. Either mutations in *FOXC1* or *PITX2* disruptions, or deletions, have been estimated to account for 6-40% of patients with ASD (estimated prevalence of 1 in 200,000 individuals,(D'haene et al. 2011) but are commonly related with systemic findings, such as dental and umbilical anomalies in patients with *PITX2* haploinsufficiency and cardiac and hearing abnormally in

patients with *FOXC1* haploinsufficiency because the proteins produced from the *PITX2* and *FOXC1* genes are transcription factors that control the activity of other genes before birth in the developing eye and in other parts of the body. Mutations in *FOXE3, PITX3, PAX6, B3GALTL, MAF, COL4A1, CYP1B1* and *SH3PXD2B* and with two known additional genetic loci (13q14, 16q24) have also been described in patients with anterior segment dysgenesis (Tümer Z & Bach-Holm D.,2009; D'haene et al. 2011; Reis et al. 2011) but there is considerable genetic heterogeneity similar to MAC and many genes remain unidentified.

1.2 Inherited retinal degeneration (IRD)

Inherited retinal degeneration diseases, are a major cause of inherited blindness across the world with a prevalence of about 1/3500 in the general population. They are clinically and genetically heterogeneous disorders characterized by progressive dysfunction of photoreceptors and vision loss. At least, 20 diagnoses can be differentiated under IRDs range from minor retinal dysfunctions to severe congenital forms of blindness (for review see Berger et al. 2010).

To date, genetic linkage studies in large pedigrees have been implicated more than 300 causative genes in different forms of retinal diseases, according to the Retinal Information Network (complete gene list available at :https://sph.uth.edu/retnet/),(see **Figure 1-2**).

IRDs distinguishable through clinical and electrophysiological through retinitis pigmentosa (RP; MIM: 268000); muscular dystrophy or Stargardt disease (STGD ;MIM :248200); and Leber congenital amaurosis (LCA ;MIM: 204000) which have been included in my dissertation. Remarkably, IRDs can be either non-syndrome or syndromes as a feature of a multisystem disorder such as, hearing loss (Usher syndrome), renal abnormalities (Senior-Løken syndrome) or polydactyly (Bardet-Biedl Syndrome, BBS) (Tiwari et al. 2016 &Sundaramurthy et al. 2016)

1.2.1 Retinitis Pigmentosa (RP)

RP has been described by primary dysfunctions of the rod photoreceptors followed by degeneration of the RPE and cone photoreceptors. Clinical hallmarks of RP are night blindness,

progressive visual field (VF) constrictions, and final loss of central vision (with a higher prevalence of 1 in 3000 to 5000 in USA and Europe). (Chiang JP & Trzupek K.,2015&Huang et al. 2015).

1.2.2 Leber congenital amaurosis (LCA)

LCA is the most severe nonsyndromic cases of the retinal dystrophy, whereas the less aggressive forms are usually considered juvenile retinitis pigmentosa. LCA, causes severe visual impairment and blindness, very early in life. Mutant alleles with at least 19 identified genes acting in different pathways, which all have critical roles for normal retinal function, were involved in the LCA development (in prevalence of 1: 30000 to 81000)(Bamshad et al. 2011 & Chiang JP & Trzupek K.,2015).

1.2.3 Stargardt macular degeneration (STGD)

Stargardt disease affects a small area near the center of the retina called the macula and is one of the most frequent causes of macular degeneration in childhood (in prevalence 1 in 8,000 to 10,000 individual), a rapidly progressive course, and finally poor visual. Although visual acuity is severely reduced, peripheral visual fields remain normal throughout life. In most cases, Stargardt macular degeneration is caused by mutations in the *ABCA4* gene. Less often, mutations in the *ELOVL4, PROM1* and *CNGB3* genes (Maugeri et al. 2002; Braun et al. 2013).

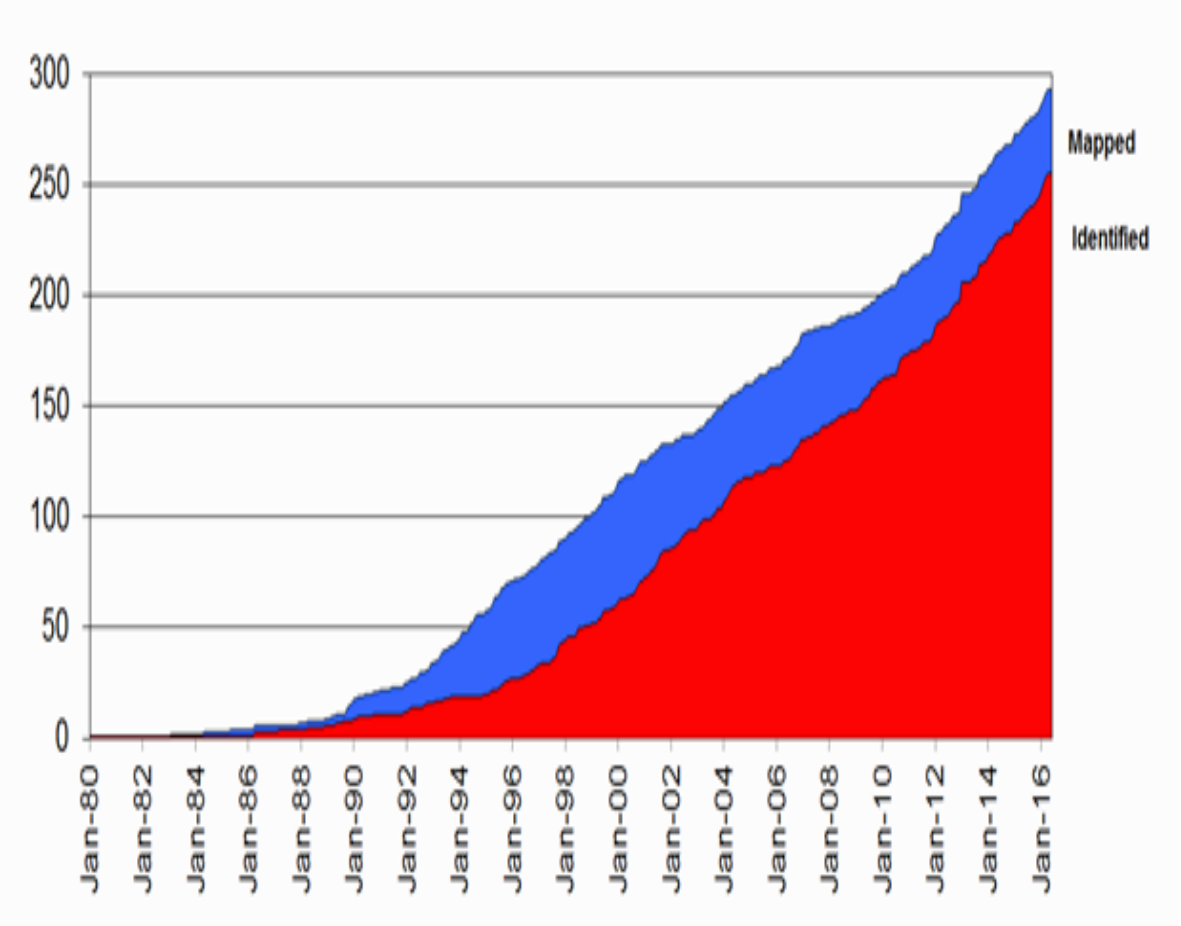

Figure 1-2: Mapped and Identified Retinal Diseases gene 1980-2016

Reprinted from RetNet, https://sph.uth.edu/retnet/sum-dis.htm.

1.3 Whole Exome Sequencing

Ocular genetics and inherited eye diseases as generally provide the greatest challenge for physicians in providing accurate diagnosis and genetic recurrence risks for family members, but also for potential therapeutic interventions such as gene therapy (if possible). (Shanks et al.2013) Until now, genetic testing technologies have been restricted to Sanger sequencing, which is very accurate, but time consuming and prohibitively expensive for large-scale sequencing, also screening methods such as the Arrayed Primer Extension (APEX) arrays which detect only known mutations. But the large number of identifying genes and inherited eye diseases makes it difficult and expensive to screen all these genes at least.

In contrast, the introduction of Next generation sequencing (NGS) has an exponential increase of revealed genetic causes in both extremely rare and common diseases, but heterogeneous disorders in an unbiased manner by sequencing the entire protein-coding sequence, known as whole exome sequencing (WES), or even the entire human genome, known as Whole genome sequencing (WGS) and both have identified new genetic causes for many conditions (Bamshad et al.2011& Neveling et al. 2013). WES is popular for two main reasons: a) the majority of known disease causing mutations are in protein coding regions of the genome and b) exome sequencing targets less than 2% of the human genome and thus is cheaper, faster and requires less computational resources than WGS.

In contrast, WGS data can be used to identify non-coding variants whose function, we will likely understand more fully with the advent of projects such as the ENCyclopedia of DNA Elements (ENCODE) and The NHGRI Genome Sequencing Program (GSP) which launched by National Human Genome Research Institute (NHGRI) (Raney et al. 2010; van et al. 2013&Wetterstrand KA.,et al 2016).

Over the last few years, the cost of NGS has been decreasing dramatically and the ability to both genotype known variants and discover novel variation across a large number of genomic loci

lead up many clinical laboratories which, have adopted NGS as an effective tool for the diagnosis of rare genetic diseases as one of the most reliable, efficient, high-throughput analysis pipeline, fast, and accurate to identify disease-causing mutations. Furthermore, it has found numerous uses in the diagnosis of heterogeneous disorders, including Ocular developmental disorders or IRD.

Introducing WES into a clinical diagnostic setting allows multiple genes to be sequenced in parallel and offers an ideal model to identify disease-causing mutations in familial cases and sporadic patients of inherited eye diseases, these could be used to improve the molecular diagnostic rates in eye disease. Recently, many researchers have shown an increased interest in to apply WES to identify disease-causing mutations, particularly for Inherited monogenic diseases of the retina (Shanks et al. 2013; Glöckle et al. 20014; Consugar et al.2015; Sundaramurthy et al. 2016; Tiwari et al. 2016a and Tiwari et al. 2016b).

Recently, the literature has emerged that offers contradictory findings about using WES in a mixture of retinal phenotypes with a "success" rates ranging from around 55-80 % (Shanks et al. 2013; Glöckle et al. 20014 and Sundaramurthy et al. 2016). Though WES fails to identify disease-causing mutations in almost 40% of the cases (Tiwari et al,2016b). There are a number different of possibilities that could explain this failure for example, (a) variants in genes not yet disease-associated (b) variants that occur outside the coding sequences and within deep intronic regions and are therefore missed by the WES, or (c) limitations of the employed method that inhibit the efficient identification of sequence alterations.

Complementary methods, such as autozygosity mapping or WGS may be considered to facilitate the identification of the disease-associated genetic alterations (Tiwari et al, 2016b)

2 Aims of the research

The general theme of this dissertation is to establish the diagnostic NGS pipeline to identify mutations in different forms of clinical heterogeneous eye defect disorders. Moreover, this study was aimed to develop a comprehensive molecular diagnostic method and to determine the efficacy, advantages and pitfalls of NGS for the rapid screening of multiple genes in unrelated and prescribed patients diagnosed with ocular developmental disorders or IRDs but unclear diagnoses in other phenotype or features in syndrome cases.

In this study, all cases were subjected to whole exome sequencing (WES), initial analysis was focused to identify variants within 450 genes associated with (MAC spectrum, ASD, RP, ALC and STGD). Other family members were also recruited to perform segregation analysis of the mutation with the disease phenotype. We present the results of cases that highlight the challenges and limitations of WES data analysis, which might have implications towards procedures used to detect mutations in gene diagnostics and research projects.

3 Materials and methods

3.1 Recruitment of Subjects and Collection of Samples.

Our work is part of a collaborative study with three different ophthalmology clinics and genetics centers, that are responsible for providing diagnostic and relative description of all the phenotype of different cases obtained from eye clinics, for basis of comprehensive Ophthalmological examination, such as functional electroretinography (ERG) and morphological studies: fluorescein angiography (FAG); optical coherence tomography (OCT) and autofluorescence imaging (AF). Then collection of the peripheral blood sample. Families (F01, F02, F06, F11) were recruited at Niguarda Ca' Granda Hospital, Milan, Italy. Families (F03, F04, F06, F07, F 08, F09, F10) at Fondazione IRCCS Policlinico San Matteo in Pavia, Italy and family (F05) at Hospital IRCCS Santa Maria Nuova Hospital in Reggio Emilia, Italy.

The selection of the number of patients that will undergo targeted NGS analysis, is based on the availability of DNA samples from both patients and/or other family members, so that segregation analysis could subsequently be performed.

We genetically screened eleven families from cohort seventeen probands were divided into two groups of syndromic diagnoses and no syndromic diagnoses with apparently isolated cases and initially with -severe un/misdiagnosed or unresolved rare genetic disorders, and their unaffected parents, participated in this study developmental eye disease seven with Coloboma/Microcornea/ Microphthalmia,five with retinitis pigmentosa, two with Leber Congenital Amaurosis, two with Stargardt Macular Dystrophy ,one with Axenfeld -Rieger anomaly were chosen for the study All the probands in the Coloboma/Microcornea/ Microphthalmia group and with Leber congenital amaurosis also the patient with Anterior Segment Dysgenesis had severe eye disease that had been identified soon after birth.

In view of the overlap in phenotypic features that may occur in these groups of hereditary ocular disease conditions and the large number of candidate disease genes, we decided to WES approach followed by test of known disease genes in these disorders. All experiments were performed in accordance with the ethical tenets of (Fondazione IRCCS Policlinico San Matteo at Department of Molecular Medicine in Pavia, Italy). All patients or family members and the parents of affected children, provided written informed consent for genetic testing.

3.2 NGS experiments

3.2.1 Sample preparation

Briefly, Total genomic DNA (around 3 µg) was extracted from peripheral blood by using GenElute™ Blood Genomic DNA Kit (Sigma Aldrich, Milan, Italy), DNA quantification was verified using the NanoDrop 1000 Spectrophotometer (EuroClone S.p.A, Milan, Italy). Then it was sheared into fragments of approximately 150-200bp by Covaris S220 instrument (Covaris, Woburn, MA, USA) to check the size of the fragments, quality control on the recovered DNA was performed by using NanoDrop 1000 spectrophotometer (EuroClone S.p.A, Milan, Italy) and 2100Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). According to the Agilent SureSelect Human All Exon version 5 kit (Agilent Technologies, Santa Clara, CA) (see **Figure 3-1**, for detailed schematic work) DNA fragments were end-repaired and purified using the magnetic bead-based Agencourt AMPure XP purification system (Beckman Coulter Genomics, Brea, CA, USA). Then we performed the extra ‘adenine’ base was added to the 3' end of the DNA fragments and the ligation of indexing-specific paired-end adapters. Modified adapters were ligated (indexing-specific paired-end adapters) to the ends of the DNA fragments and six (few) cycles of PCR amplification were applied to each sample after ligation, 750 ng of DNA from the resulting libraries were hybridized to the bait set using the Agilent SureSelectXT Capture Library Kit at 65°C for 24 h. Hybrids capture was performed according to the manufacturer’s protocol with Streptavidin-coated Dynal magnetic beads (Invitrogen, Carlsbad,

CA, USA). Captured samples were further purified through the Agencourt AMPure XP beads and subjected to a PCR-based amplification reaction to add unique 6 bp-index tags and 8-samples pool together, accordingly to Agilent SureSelect Human All Exon v5 protocol. Finally, the multiplexed libraries were loaded to sequence on HiSeq2500 using 100 bp paired-end reads to capture consensus coding sequence exonic regions and flanking intronic regions totaling ~50 Mb of genomic DNA according to the manufacturer's instructions (Illumina, San Diego, CA, USA).

3.2.2 Whole exome sequencing (WES) analysis

Extracted data were aligned against the GRCh37/hg19 human genome, indexing of the reference genome, variant calling and annotation was achieved using a pipeline based on Burrows-Wheeler Aligner (BWA), is a software package for mapping low-divergent sequences against reference genomes, for example the human genome. Variant calling and assembly of variant files were performed using standard procedures with software such as Samtools and Picard, which produce binary formats (BAM) from raw data. Since hereditary ocular diseases are rare in the population, we considered variants with a frequency above 1% in control population databases (Exome Variant Server EVS, ESP-6500; 1000g and dbSNP 138) as benign polymorphisms, providing the cohort size was sufficiently diverse and large, and the sequencing read depth exceeded an average of 70×.

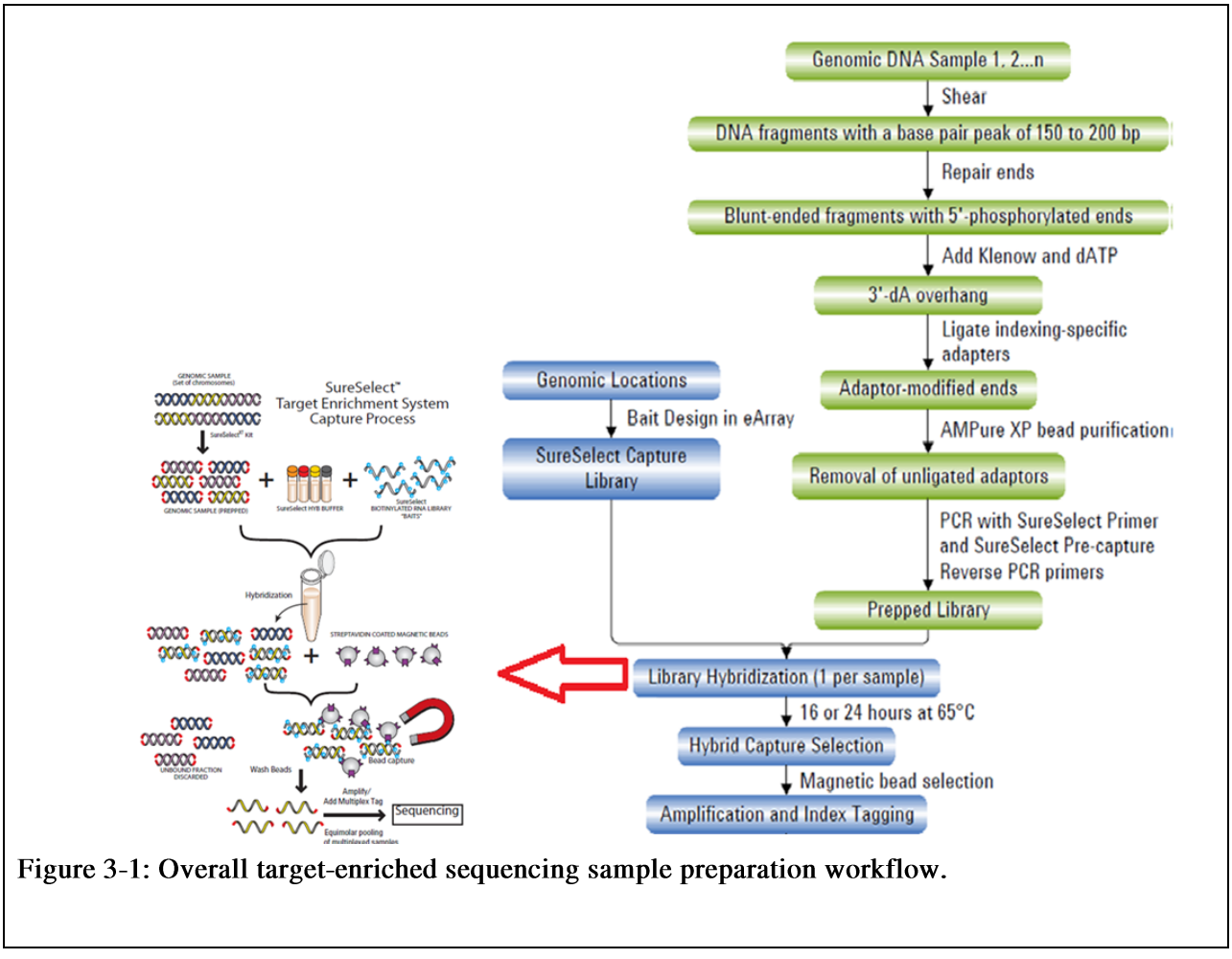

Figure 3-1: Overall target-enriched sequencing sample preparation workflow.

3.2.3 Annotation, interpretation of data and Filtering approaches

To narrow candidate variants, we listed the candidate genes for each symptom and clinical diagnosis (**Table 3-1**). Clinical interpretation was narrow to variants within coding regions ±20 bp of these genes. The pathogenicity of the remaining genetic variance with frequency below 1% in SNP databases that have been described in scientific literature, the patient's clinical referral (genotype and phenotype correlations) and the Human Gene Mutation Database (HGMD) to be disease-associated were given higher priority.

In the variant types, protein truncation mutations leading to loss of function or structure (nonsense or frameshift) were given higher priority. Pathogenicity of non-truncation mutations (missense) were tested by at least one of the three silico modelling (computational programs) protein prediction algorithms SIFT, PolyPhen2 and MutationTaster2 (Richards et al. 2015).

A manual inspection by using Integrative Genomics Viewer (IGV) on the BAM files, allowed us to evaluate the coverage, quality of the aligned reads and to identify potential insertions or deletions in our interest regions. We also using a novel software package called EXCAVATOR to detect of copy number variations (CNVs) from WES data (Magi et al. 2013)

3.2.4 Variant validation (Sanger sequencing analysis)

We achieved PCR and bidirectional capillary sequencing to confirm the Zygosity of most likely disease-causing variants and carrier variants in the patient and the available family members before they were clinically reported. All target regions were amplified and PCR reactions were carried out with the GoTaq G2 Flexi DNA polymerase kit (Promega, USA) in a final volume of 25 µl containing 50 ng of genomic DNA, 5X Green GoTaq® Flexi Buffer, 25mM MgCl2, and 0.2mM each dNTP, 5u/µl. of GoTaq G2 Flexi DNA polymerase and 10 pmol of both forward and reverse primers (**Table 3-2**) . Primers were designed using Primer 3 Plus and purchased at sigma-aldrich(Milan-italy). DNA samples were denatured at 95°C for 5 min and then amplified for 35 cycles as the following, 95°C for 30 sec: annealing at variable annealing temperatures (see table 2) :72°C for 30 Sec; final extension at 72°C for 7 min. PCR products were purified by SigmaSpin™ Sequencing Reaction Clean-Up (Sigma-Aldrich,USA). Sequencing reactions were performed using the ABI BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, California, USA), then sequencing reaction products were purified by DyeEx 2.0 Spin Kit (Qiagen, USA) and ABI 3730 DNA Analyzer (Applied Biosystems , California, USA). Sanger sequencing data analysis was achieved using the Sequencing Analysis Software v5. Phenotypic demonstration and evidence for variant pathogenicity was debated in detail within a clinical

multidisciplinary team meeting (if applicable). Additional testing was asked for other family members to assist with the interpretation of mutant pathogenicity and to clarify the risks to additional family members.

3.2.5 Qualifying variants in genes already implicated in similar phenotypes

For each qualifying gene in patients that have severe undiagnosed rare genetic disorders, and their unaffected parents, participated in this study, we considered whether it had been associated with a closely matching phenotype by searching the Online Mendelian Inheritance in Man (OMIM) database and PubMed. When the genes were involved, we then asked whether the precise mutations were reported in the literature to cause a closely matching phenotype, and if not, whether the mutations have been evidently within the same class as already known disease causing mutations (e.g., dominant, recessive).

Table 3-1: Overview of the gene list of our method

To obtain a complete list of genes associated with IRD or Ocular developmental disorders, we performed comprehensive searches in the most important databases were taken as supplementary sources, which are professional HGMD, Leiden Open Variation Database 3.0 (LOVD3.0), Retinal Information Network, RetinoGenetics database, Online Mendelian Inheritance in Man (OMIM) and PubMed database for related publications.

ABCA4	BBS12	CEP164	CORS2	F13B	GPR125	INPP5E	MCDR3	NT5C1B	PDZD7	PRPF8	ROM1	SMOC1	TREX1	XRCC1
ABCB6	BBS2	CEP250	COX7B	FAM120A	GPR179	INVS	MDDC	NTF4	PEX1	PRPH2	RP1	SNRNP200	TRIM32	YAP1
ABCC6	BBS4	CEP290	CRB1	FAM161A	GPR98	IQCB1	MERTK	NYX	PEX2	PRPS1	RP15	sox10	TRPM1	ZNF423
ABHD12	BBS5	CERKL	CRB2	FAM27L	GRIP1	ISPD	MFN2	OAT	PEX26	PRSS56	RP1L1	SOX2	TSPAN12	ZNF513
ACHM1	BBS7	CETP	CRX	FBLN5	GRK1	ITM2B	MFRP	OFD1	PEX7	PXDN	RP2	SPATA7	TTC8	ZNRF1
ACVR1C	BBS9	CFB	CRYBA4	FBN1	GRM6	JAG1	MIR204	OLFM2	PGAM5	RAB18	RP22	SPP2	TTLL5	
ADAM9	BCOR	CFH	CSNK2A2	FHASD	GSTM1	KARS	MITF	OPA1	PGK1	RAB28	RP24	STAT6	TTPA	
ADAMTS18	BEST1	CFHR2	CSPP1	FKRP	GUCA1A	KCNJ13	MKKS	OPA2	PHIP	RAB3GAP	RP29	STRA6	TUB	
ADAMTSL3	BMP4	CFHR4	CX3CR1	FKTN	GUCA1B	KCNV2	MKS1	OPA3	PHYH	RAB3GAP	RP32	TAL1	TUBGCP4	
ADGRA3	BTAF1	CFHR5	CYP1B1	FLVCR1	GUCY2D	KIAA1549	MMP9	OPA4	PITPNM3	RAD54B	RP34	TBC1D20	TUBGCP6	
ADGRV1	C12orf57	CFI	CYP46A1	FNBP4	HARS	KIF11	MRST	OPA5	PITX2	RARB	RP5	TBK1	TULP1	
AHI1	C12orf65	CHD7	CYP4V2	FOXC1	HCCS	KIZ	MTTP	OPA6	PITX3	RAX	RP6	TCF7L1	TULP3	
AIED	C1QTNF5	CHM	DACH1	FOXE3	HESX1	KLHL7	MVK	OPN1LW	PKD1	RAX2	RP63	TCF7L2	UNC119	
AIPL1	C2	CIB2	DAG1	FRAS1	HEY1	LAMA1	MYO7A	OPN1MW	PLA2G5	RB1	RP9	TEAD1	USH1A	
ALDH1A3	C21orf2	CLN3	DFNB31	FREM1	HGSNAT	LARGE	MYOC	OPN1SW	PLK4	RBP3	RPE65	TECTA	USH1C	
ALMS1	C2orf71	CLRN1	DHDDS	FREM2	HK1	LCA5	NAA10	OPTC	PNPLA6	RBP4	RPGR	TENM1	USH1E	
ALX1	C3	CNGA1	DHX38	FSCN2	HMCN1	LHFPL5	NAB2	OPTN	POC1B	RCD1	RPGRIP1	TENM3	USH1G	
ANO7	C8orf37	CNGA3	DMD	FZD4	HMGB3	LIPC	NDP	orf15	POMGNT1	RCD2	RPGRIP1L	TFAP2A	USH1H	
APOE	CA4	CNGB1	DMXL1	GAL3ST3	HMX1	LOXHD1	NEK2	OTX2	POMK	RD3	RS1	TIMM8A	USH2A	
ARL2BP	CABP4	CNGB3	DRAM2	GALC	HTRA1	LOXL1	NEUROD1	PAK7	POMT1	RDH11	SAG	TIMP3	USH2B	
ARL6	CACD	CNNM4	DSPP	GATA6	IDH3B	LRAT	NHS	PANK2	POMT2	RDH12	SALL1	TLR3	VAX1	
ARMS2	CACNA1F	COD2	EDA	GDF3	IFT140	LRIT3	NIPBL	PAX2	PORCN	RDH5	SDCCAG8	TLR4	VCAN	
ASB10	CACNA2D	COL11A1	EFEMP1	GDF6	IFT172	LRRC4	NMNAT1	PAX6	PRCD	RECQL4	SEMA4A	TMEM126A	VMD1	
ASPM	CAPN5	COL2A1	ELOVL4	GIPC3	IFT27	LTBP2	NOS3	PCDH15	PRD	RGR	SH3PXD2B	TMEM237	VSX2	
ATF6	CASK	COL4A1	ERCC2	GJA1	IGBP1	LZTFL1	NPHP1	PCYT1A	PRDM13	RGS9	SHH	TMEM5	WDPCP	
ATXN7	CC2D2A	COL4A6	ERCC5	GNAT1	IKBKG	MAB21L2	NPHP3	PDE6A	PROM1	RGS9BP	SIX3	TMEM67	WDR19	
B3GALNT2	CD5	COL8A2	ERCC6	GNAT2	IMMT	MAF	NPHP4	PDE6B	PRPF3	RHO	SIX6	TNC	WDR36	
BBIP1	CDH23	COL9A1	ERCC8	GNB5	IMPDH1	MAK	NR2E3	PDE6C	PRPF31	RIMS1	SLC24A1	TNF	WFS1	
BBS1	CDH3	CORD1	EVR3	GNPTG	IMPG1	MAP2	NR2F1	PDE6G	PRPF4	RLBP1	SLC4A3	TOPORS	WFS2	
BBS10	CDHR1	CORD8	EYS	GPR12	IMPG2	MAP6	NRL	PDE6H	PRPF6	RNANC	SLC7A14	TP53	XKR4	

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Table 3-2: Sequences and characteristics of primers used for mutation analysis.

Tm: melting temperature; Ta: annealing temperature . Tm: comes from Primer3Plus

Primer name	Sequence (5`>3`)	Tm (°C)	Ta (°C)	Amplicon Size(bp)	chromosomal coordinator (GRCh37/hg19)
RAB3GAP1-ex25-F	CATCTGTCCTTCAAAAGCATC	57.4	59.0	377	chr2:135926043+135926419
RAB3GAP1-ex25-R	GGTACCTTCCCTCCCTCAG	58.5			
STRA6-ex19-F	GATCAGGTCTGAGGGCCAG	60.8	59.0	302	chr15:74472356-74472657
STRA6-ex19-R	GAGGAGGATGGTAGGCAGG	59.6			
CHD7-ex17-F	AAGGAGCAAGTATGTTGTCGC	59.4	62.0	330	chr8:61749316+61749645
CHD7-ex17-R	TGACTGGTACTCTCTGTCATAGTAAGC	59.5			
KCNK9-ex2-2-F	CATAGTCCTGCGAGCGGTAG	60.9	60.0	279	chr8:140630704+140630982
KCNK9-ex2-2-R	AGAAGAAGCCGCTCTACGTG	59.8			
GUCY2D-ex2-F	GTCCCCGCTTCGAGGTAG	60.8	61.0	538	chr17:7906643+7907180
GUCY2D-ex2-R	CATGATCACTGCTGCGGAC	61.5			
CDH23-ex25-F	GCTCTGGAGCTGGGTCTTC	60.0	60.0	357	chr10:73466589+73466945
CDH23-ex25-R	CAGAGGCTAAAGCCCAACAG	60.1			
CDH23-ex31-F	GGCAGCTTGAGAAGCCAC	59.7	59.0	463	chr10:73491670+73492132
CDH23-ex31-R	CGTGATGGCGTCTATCTTGA	59.8			
USH2A-ex55-F	GGATAAGTGGGACCTGACCA	59.8	59.0	691	chr1:215953038+215953728
USH2A-ex55-R	GATGGGCCATATGGAAGAGA	59.9			
USH2A-ex6-F	GGCATTTGTTGCAATAACCA	59.4	59.0	371	chr1:216498591+216498961
USH2A-ex6-R	CTCCCTCTTGACTCCCACAG	59.8			
TULP1-ex8 -F	TAGGCTCCCAAGTCCAGG	58.7	59.0	231	chr6:35476913+35477143
TULP1-ex8 -R	ACTTTGCAAACCAGGGTGAG	60.1			
TULP1-int8-F	GTACATCAGCCCCAGAGCAC	60.7	60.0	211	chr6:35473968+35474178
TULP1-int8-R	CTCCCCAGAGCCTCCTAACT	59.8			
FOXC1-ex1-F	GCGCATCCAGGACATCAAGA	64.6	60.0	206	chr6:1611343+1611548
FOXC1-ex1-R	AATCCGCACCGTCCAGGC	66.8			
ABCA4-ex36-F	CTGGTCCTTCAGAGCACACA	60.0	59.0	349	chr1:94485063+94485411
ABCA4-ex36-R	GGTGTAAGGCCTTCCCAAAG	60.0			
GPR98-ex23-F	TCATTGCTTAGTGCCTCTGG	59.0	59.0	240	chr5:89969835+89970074
GPR98_ex23-R	TTCTTGCAAAAGGAGCAGGT	60.0			
CNGB3-UTR3-F	CATCAGGAAAGAACCAAAGGA	59.0	59.0	389	chr8:87587747+87588135
CNGB3-UTR3-R	CCAGAGGGACTTCTCGTCAA	60.0			

4 Results

4.1 Clinical details

We performed diagnostic NGS testing in 16 patients (6 males and 10 females) referred with clinical indications of inherited eye diseases. All pedigrees of the families that have been participating in the study are shown in (**Figure 4-1**). Detailed clinical phenotypes of 11 families and 16 patients are provided in the clinical descriptions of patient section in (**Table 4-1**). The majority of analyzing patients (n = 16) originated from Italy. We cannot, however, exclude that some patients have migrated to Italy.

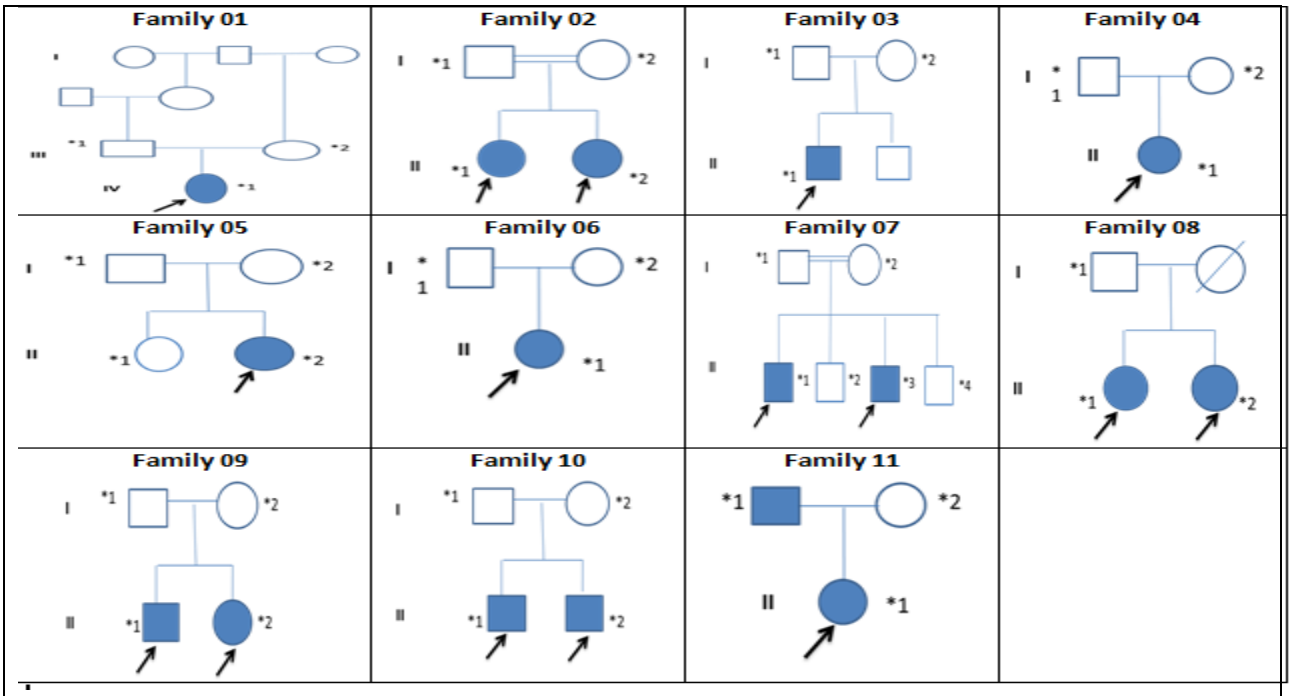


Figure 4-1: Pedigrees of the families studied

A proband from each of the families underwent exome sequencing. Probands are indicated by arrows. The asterisk indicates DNA sample available. The solid squares (male) and circles (female) represent affected patients, the doubled line indicates consanguineous marriage. The generation number is shown on the left.

Table 4-1: Clinical features of all cases study

	FAMILY 01	FAMILY 02	FAMILY 03	FAMILY 04	FAMILY 05	FAMILY 06	FAMILY 07	FAMILY 08	FAMILY 09	Family F10	FAMILY 11
Consanguinity	YES	YES	NO	None	NO	NO	Yes	NO	NO	NO	NO
Family history	None	Mone	None	None	None	None	None	None	None	None	Father
Origin of patient	Italian	Moroccan	Italian	Italian	Italian	Italian	Moroccan	Italian	Italian	Italian	Italian
Recruitment site	Milan	Milan	Pavia	Pavia	Emilia-Romagna	Milan	Pavia	pavia	Pavia	Pavia	Milan
Gender	Female	Female/Female	Male	Female	Female	Female	Male/Male	Female/Female	Male/Female	Male/Male	Female
Age (year)	2Y	8M & 8Y	7Y	2Y	8Y	12Y	1Y&5Y	26Y&33Y	12Y&13Y	20Y&26Y	2Y
number of affected individual taken for analysis	One	Two	One	One	One	One	Two	Two	Two	Two	One
Ocular Phenotypes)	Coloboma/Microcornea/ Microphthalmos Optic nerve hypoplasia Congenital cataract Congenital primary aphakia	Coloboma/Microcornea/ Microphthalmos	Retinite Pigmentosa	Coloboma/Microc ornea/ Microphthalmos Chorioretinal with involvement of the macula	Microphtalmia/Coloboma	Coloboma/Microcornea/ Microphthalmos atrophy & hemorrhag Retinal Vitreoretinal abnormalitie	Leber congenital amaurosis	Retinite Pigmentosa	Retinite Pigmentosa	Macular Dystrophy	Anterior Segment Dysgenesis
Additional features	Neurodevelopmental delay, Psychomotor retardation, Congenital microcephaly, Generalized hypotonia , Microgenia, hint bilateral fifth finger clinodactyly,distal phalanges, Nares Anteverted, straight eyebrows	congenital heart defect (PA-VSD & TOF) with interrupted aortic arch only in older sister	hearing impairment	Retardation of growth and development,congenital heart defect	Delayed psychomotor development,Low birth weight, Feeding difficulties,ectopic kidney,Congenital Heart Disease, hearing problem,Cleft palate,Short philtrum,Flared eyebrows	Global developmental delay, Generalized hypertrichosis, Quadriplegia,Microcephaly, sevre neuro sensory hearing impairmen, Anteverted nares, verrucous patches with Blaschko lines , thick hair and eyebrows , abnormal dental development	_____	_____	_____	_____	_____
suspected disorder	Cerebrooculofacioskeletal syndrome or Xeroderma pigmentosum, group G/Cockayne syndrome	Microphthalmia syndromic	Usher Syndrome	Microphthalmia syndromic	Microphthalmia syndromic	Walker Warburg syndrome (WWS) or muscle-eye-brain disease (MEB), Warburg micro syndrome, Incontinentia pigmenti (IP), Cornelia de Lange syndrome (CDLS)	Leber congenital amaurosis	Retinite Pigmentosa	Retinite Pigmentosa	Stargardt Macular Dystrophy	Anterior Segment Dysgenesis
diagnosis before NGS	syndromic	syndromic	syndromic	syndromic	syndromic	syndromic	non syndromic	non syndromic	non syndromic	non syndromic	non syndromic

4.2 The results of Exome Sequencing.

Whole-exome sequencing resulted with an average (85-87%) of all targeted regions being covered by(≥15X) reads and the average coverage >70X.All identified mutations and variants description and pathogenicity predictions of these sequence variations are shown in (**Table 4-2**). Mutations are named according to HGVS nomenclature guidelines (www.HGVS.org). We identified putative disease-associated variants in 13 cases out of 16 cases. Positive diagnoses were observed in 9 out of 11 families (4 syndromic diagnosis groups and 5 non- syndromic diagnosis groups).

Table 4-2: Causative variants—allele frequencies in public databases, conservation, and prediction categories.

Abbreviations: **D**, damaging; **T**, tolerated; **NA**, Not Available; **B**, benign; **P**, polymorphism; **MAF**, Minor allele frequency. **ExAC**,Exome Aggregation Consortium;***RAB3GAP1*** :RAB3 GTPase Activating Protein Catal.ytic Subunit 1;***STRA6***:Stimulated By Retinoic Acid 6;***CDH23***:Cadherin-Related 23 ***CHD7***:Chromodomain Helicase DNA Binding Protein 7;***KCNK9***:Potassium Two Pore Domain Channel Subfamily K Member 9; ***GUCY2D***: Guanylate Cyclase 2D;**USH2A** :Usher Syndrome 2A;***TULP1***:Tubby-related protein 1 ;***ABCA4***:Retinal.-specific ATP-binding cassette transporter; ***GPR98***:G-protein coupled receptor 98;***CNGB3***:Cyclic nucleotide-gated cation channel beta-3

Sample (family, pedigree ID)	Gene symbol	MIM number	Exon/ Intron	Nucleotideal.teration and prediction Protein al.teration	Het/ Homo state	Novel (dbSNP/1000 Genomes)	al.lele frequencies in public database (MAF)	Supporting evidence from literature (referance)	Prediction tools (SIFT,Polyphen-2, MutTaster
F01, IV.1	RAB3GAP1	NM_001172435.1	Ex 25	Chr2:135926258C>G:c.2874C>G :p.Tyr958*	homo	YES	_____	This study	Stop
F02 ,II.1 & II.2	STRA6	NM_001142617.1	Ex 19	Chr15:74472461C>T:c.1964G>A:p.Arg655His	homo	rs397514639	T=0.00002/2 (ExAC)	HGMD(CM097111); Segel et al. 2009	D,D,D
F03, II.1	CDH23	NM_001171930.1	Ex 31b	Chr10:73492055C>T:c.4027C>T:p.Leu1343Phe	het	YES	Not	This study	D,D,D
		NM_001171931.1	Ex25	Chr10:73466834G>T:c.3134G>T:p.Cys1045Phe	het	rs749852928	T= 0.00000833/1(ExAC)	This study	T,B,P
F04, II.1	CHD7	NM_017780.3	Ex 17	Chr8:61749425del:c.4039del:p.al.a1347Glnfs*25	het	YES	_____	This study	Frameshift
F05,II.2	KCNK9	NM_001282534.1.	Ex 2	Chr8:140630830C>T:c.796G>A:p.Gly266Arg	het	rs370302731	T=0.000008/1 (ExAC)	This study	T,B,D
F07, II.1 &II.3	GUCY2D	NM_000180.3	Ex 2	Chr17:7906754delC:c.387delC:p.Pro130Leufs*36	hom	rs61749670	_____	HGMD (CD962030); Perrault et al. 1996	Frameshift
F08, II.1 &II.2	USH2A	NM_206933.2	Ex 6	Chr1:216498750T>C:c.1040A>G: p.Asp347Gly	het	_____	_____	HGMD(CM1413399);Sodi et al.2014	D,D,D,
		NM_206933.2	Ex55	Chr1:215953307A>G:c.10817T>C: p.Leu3606Pro	het	_____	_____	HGMD(CM104143);Retinal.MD_039 61 ;McGee et al. 2010	D,D,D,
Fo9, II.1 &II.2	TULP1	NM_003322.4	Ex8	Chr6:35477011:c.797G>T:p.Gly266Val.	het	rs150480343	A=0.0008/93 (ExAC)	This study	D,D,D
			Int8	Chr6:35474073 C>G:c.823-17G>C	het	rs12215920	G=0.3447/10890 (ExAC)	This study	
F10, II.1 &II.2	ABCA4	NM_000350.2	Ex 36	chr1:94485247 C>T :c.5087G>A:p.Ser1696Asn	het	rs61750564	T=0.00002/2 (ExAC)	HGMD (CM990061) ;(Retinal.MD_00069);Lewis et al.1999	D,D,D
	GPR98	NM_032119.3	Ex23	chr5:89969880 A>G :c.4939A>G:p.Ile1647Val.	het	rs72782753	G=0.0048/514 (ExAC)	_____	T,B,P
	CNGB3	NM_019098.4	UTR3	chr8:g.87588003C>A:c.2507G>T		rs138432513	A=0.0036/418 (ExAC)	_____	NA,NA,P
F11, II.1	FOXC1	NM_001453.2	Ex1	Chr6:1611456 ins C: c.780dup: p.Asp261Argfs*45	het	_____		HGMD (CI117370); D'haene et al.2011	Frameshift

4.2.1 Syndromic inherited eye diseases

4.2.1.1 Autosomal Recessive

FAMILY 01: Coloboma/Microcornea/ Microphthalmia (RAB3GAP1):

A novel homozygous nonsense mutation was detected in RAB3GAP1 (NM_001172435.1, c.2874C>G, p. Tyr958*); located in exon 25 of the RAB3GAP1 gene in the patient. The mRNA produced might be targeted for nonsense mediated decay (NMD). Sanger sequencing confirmed the presence of the variant identified by WES in the patient as homozygous and both unaffected consanguineous parents is heterozygous carriers of the mutation (**Figure 4-2**). Mutations in this catalytic subunit gene, doesn't only explain the developmental eye disease, but also are associated with the Warburg micro syndrome (WARBM1; MIM 600118), which is a rare autosomal recessive syndrome characterized by, microphthalmia, microcornea, congenital cataracts, optic atrophy, cortical dysplasia, in specifically corpus callosum hypoplasia, microcephaly, severe mental retardation, spastic diplegia, and hypogonadism (for summary see Morris-Rosendahl et al. 2010). The findings strongly indicated that *RAB3GAP1* is essential for normal eye and brain development. Asahina and his colleagues have written the most complete review of the *RAB3GAP1* mutations reported to date they reported the total of 69 different mutations of various types (nonsense/missense, frameshift, and splice site mutations) have been identified in *RAB3GAP1* of 65 families (Asahina et al. 2016)

FAMILY 02: Coloboma/Microcornea/ Microphthalmia (*STRA6*):

In consequence to our discrete filtering approaches, we were able to identify in a homozygous missense mutation (c.1964G>A: p. Arg655His; RefSeq accession number (NM_001142617.1), a transition in exon 19 of *STRA6* in both affected sisters of the family (F02). It represents a rare variant (rs397514639) that was detected heterozygously in 2 out of 121,406 individuals (MAF = T=0.00002/2 (ExAC)). And reported pathogenic by HGMD (CM097111) as microphthalmia, syndromic 9; MIM 601186. .Segel et al.2009 reported children girl with clinical anophthalmia,

bushy eyebrows, patent ductus arteriosus, and normal motor and cognitive development, who was compound heterozygous for 2 missense mutations in the *STRA6* gene, one of these mutations is the same which we identified but in homozygosity state. This variant was confirmed by Sanger in all affected family members (the patient and her sister), unaffected consanguineous parents were both heterozygous carriers for the mutation (**Figure 4-3**).

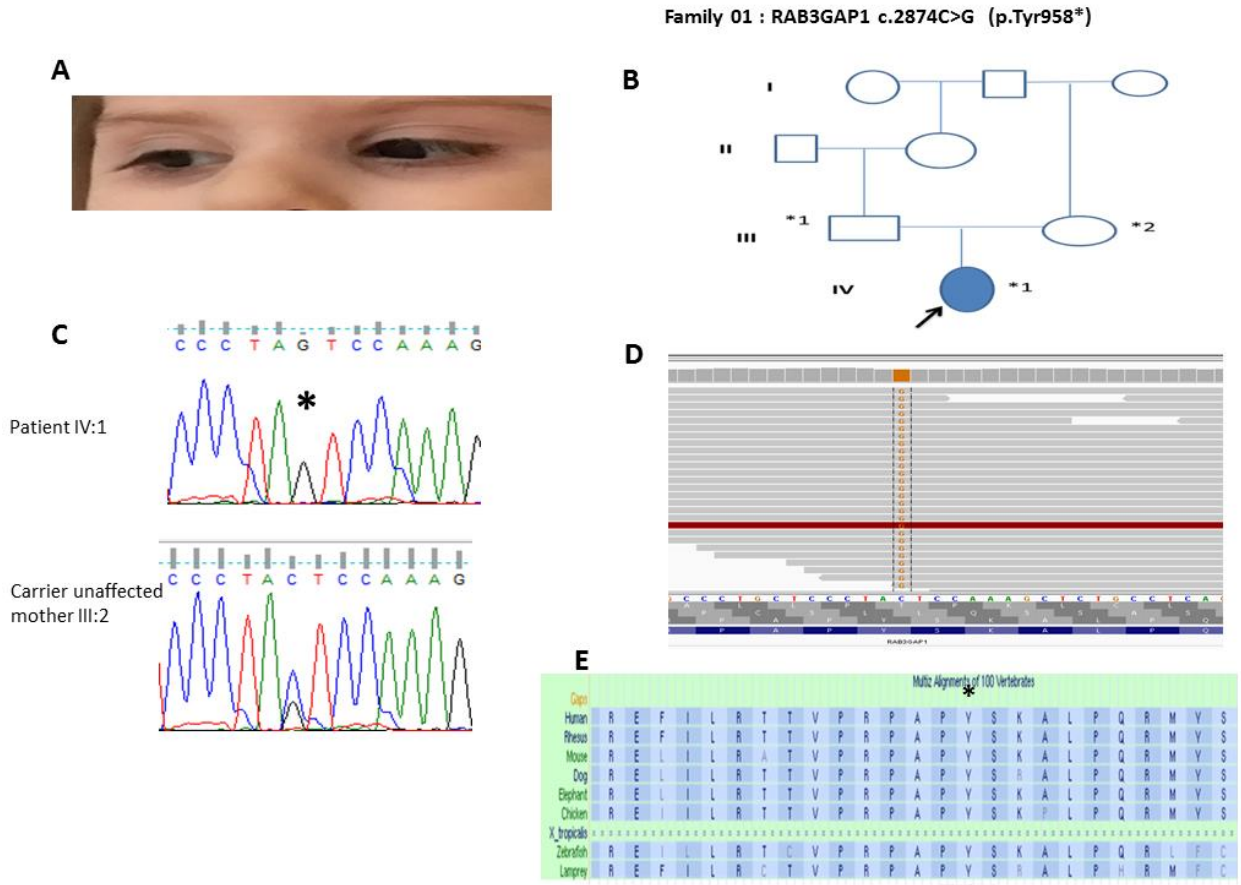


Figure 4-2: Exome sequencing identified a novel homozygous mutation in *RAB3GAP1* in the family (F01)

(A) Photographs of nuclear Coloboma/Microcornea/ Microphthalmia in patient II.1. (B) Pedigree of consanguineous family segregating of eye defect and other distinct phenotypes (see Table 4-1 for details). The arrow indicates the index case analyzed by next generation sequencing.(C) Sequence chromatograms for mutations are shown with the corresponding normal chromatograms below for comparison. Homozygous mutations are indicated by the asterisks (*). (D) Detailed view of individual sequencing reads (horizontal gray bars), as visualized by Integrative Genomics Viewer (IGV). (E) Exome sequencing identified novel *ARAB3GAP1* homozygous mutations replacing a highly conserved residue (Tyr) at exon 25 as shown in the multispecies up to Tetraodon (considering 13 species) and paralog alignment.

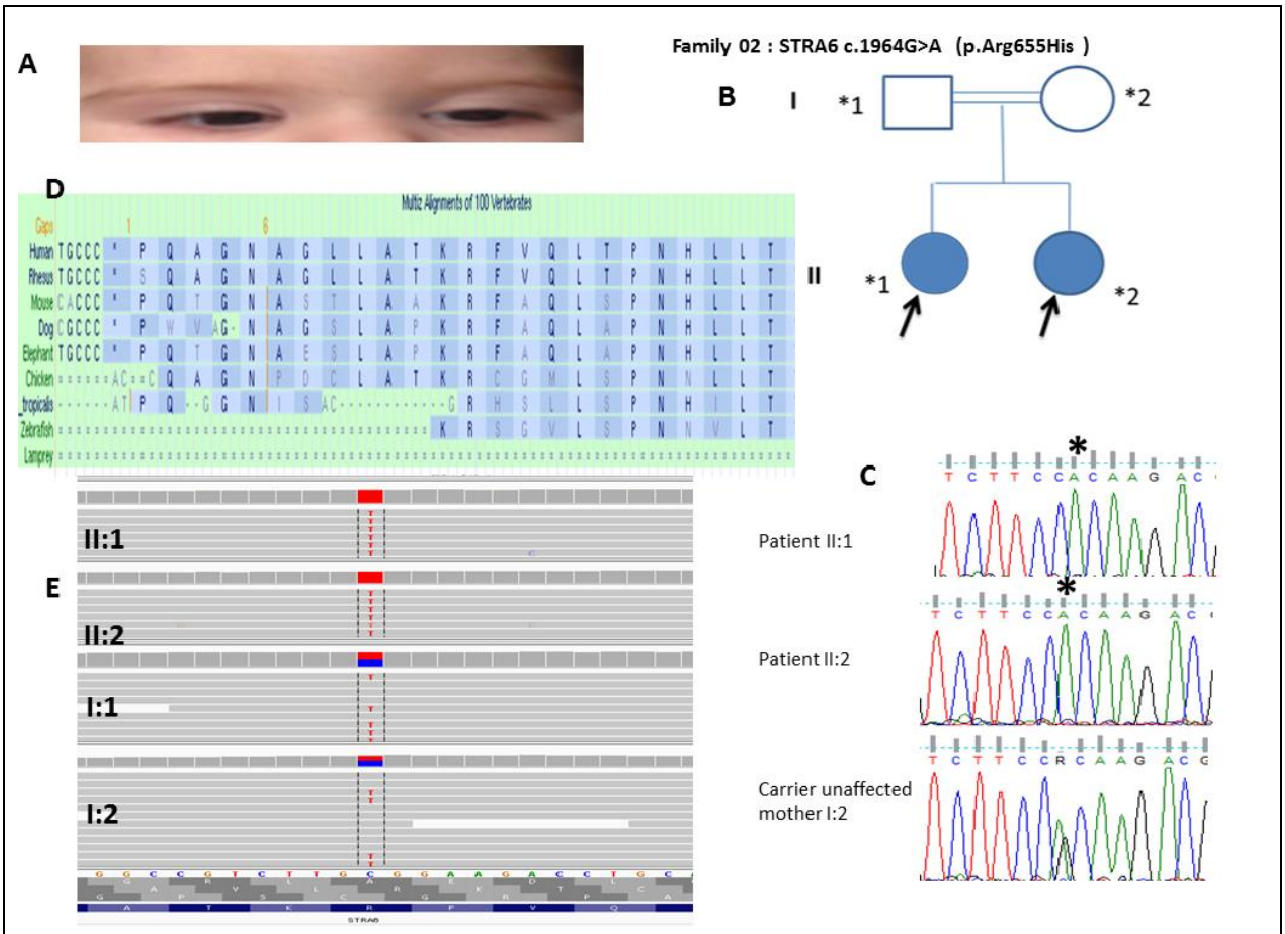


Figure 4-3: Exome sequencing identified a novel homozygous mutation in *STRA6* in the family (F02)

(A) Photographs of nuclear Coloboma/Microcornea/ Microphthalmia in patient II.2. (B) Pedigree of consanguineous family segregating of eye defect and other distinct phenotypes (see Table 4-1 for details). The arrow indicates the index case analyzed by next generation sequencing. (C) Sequence chromatograms for mutations are shown with the corresponding normal chromatograms below for comparison. Homozygous mutations are indicated by the asterisks (*). (D) Exome sequencing identified novel *STRA6 homozygous* mutations replacing a highly conserved residue (Arg) at exon 19 as shown in the multispecies and paralog alignment. (E) Detailed view of individual sequencing reads (horizontal gray bars), as visualized by Integrative Genomics Viewer (IGV).

FAMILY 03: Usher Syndrome Type I (CDH23):

We found two missense heterozygous mutations in CDH23 gene NM_001171930.1 (c.4027C>T: p. Leu1343Phe) and (c.3134G>T: p. Cys1045Phe) . The first variant was not reported in dSNP and was confirmed inherited from his father, but the second variant represents extremely rare variant (rs749852928), that was detected heterozygously in 1 out of 120084 individuals and was confirmed inherited from his mother (**Figure 4-4**). The case had profound congenital non-progressive deafness and retinitis pigmentosa and diagnostic as Usher syndrome type 1 (USH1, MIM 276900).

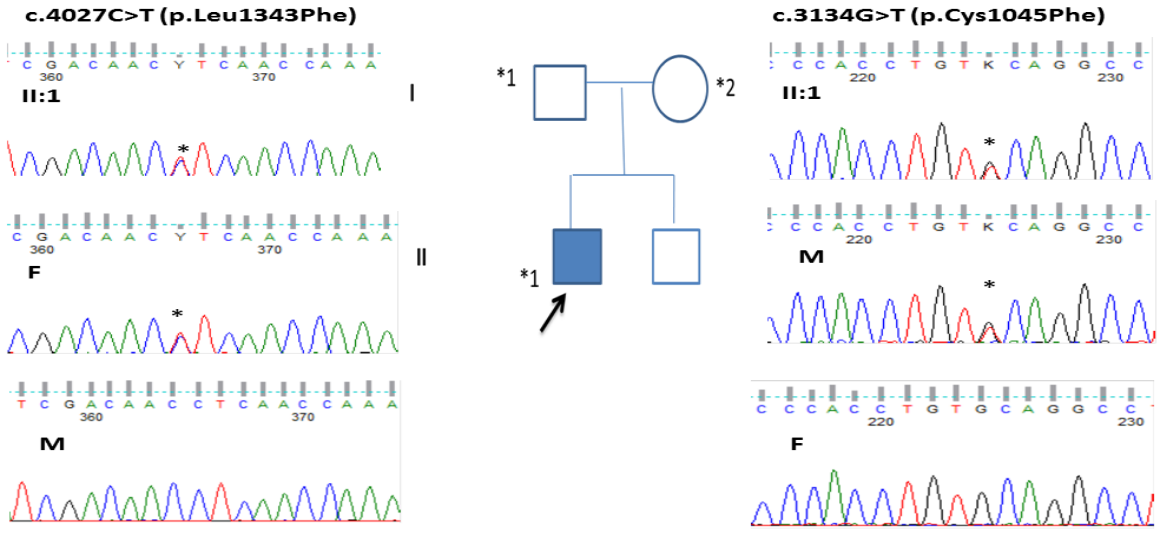


Figure 4-4: Family pedigree and transmission of the Compound heterozygouse mutations (c.4027C>T and c.3134G>T) in the CDH23 gene in the family F03

Sanger sequencing electropherograms for both loci are shown in the Left panel: Exon 31 region missense mutation: c. 4027C>T (p. Leu1343Phe). The sequences for both the Father (F) and II:1 son show heterozygosity with C and T at the same location, the asterisks (*), while the mother's sequence is normal (M). Right panel: Exon 25 region missense mutation: c. 3134G>T (p. Cys1045Phe). The sequences for both the mother (M) and II; 1 son show heterozygosity with G and T at the same location, the asterisks (*) while the fathers sequence is normal.

4.2.1.2 Autosomal Dominant

FAMILY 04: Coloboma/Microcornea/ Microphthalmia (*CHD7*):

We found a de novo heterozygous deletion (1 bp) in *CHD7* gene (NM_017780.3) in exon 17. This deletion creates a frameshift starting at codon Ala1347 (**Figure 4-5**). The new reading frame ends in a STOP codon 24 positions downstream (c.4039del, p. Ala1347Glnfs*25). The mRNA produced might be targeted for nonsense mediated decay (NMD). Mutations in this gene not only explain the developmental eye disease, but also explain congenital anomalies including choanal atresia, malformations of the heart, and retardation of mental and somatic development. *CHD7* mutations, are present in 65–70% of patients clinically diagnosed with CHARGE syndrome (MIM 214800). In an estimated 1:10,000 births worldwide, named by the acronym summarizing the six major clinical features: Coloboma of the eye, Heart defects, Atresia of the choanae, Retardation of growth and development, and Ear abnormalities and deafness. Those features are no longer used in making a diagnosis of CHARGE syndrome, but still not changing the name (Zentner et al. 2010; Busa et al. 2016 &Kohmoto et al. 2016).

FAMILY 05: Coloboma / Microphtalmia (*KCNK9*):

Before using NGS approach, a traditional Sanger sequencing analysis and Multiplex ligation-dependent probe amplification (MLPA) of *SOX2, CHD7, PAX6,* and *OTX2*, was performed in patient F05;II:2 because of suspected microphthalmia syndromic. However, the analysis did not detect any causative mutation. Subsequently, for the detection of de novo mutations, a trio analysis is necessary, including the affected child and both healthy parents, WES analysis detected a cumulative total of 139,048 variants with a mean coverage > 80X. Our filtering criteria were satisfied by three variations (*HERC2, MAN2B* and *KCNK9*). Which subsequently were validated by Sanger sequencing to confirm and determine whether the candidate variant co-segregated with the phenotype within the pedigree. *HERC2* gene *MAN2B* was false positive, only *KCNK9* confirmed with Sanger (**Figure 4-6**). We found heterozygous G-to-A transition at

nucleotide 796 in exon 2 of the *KCNK9* gene, at codon 266 (p.Gly266Arg): NM_001282534.1. It represents a rare variant (rs370302731) that was detected heterozygously in 1 out of 121,412 individuals in the EVA_EXAC and NHLBI-ESP (MAF = T=0.000008/1 (ExAC) in European Americans exclusively because it was not found among African Americans. A mutation in *KCNK9* (p.Gly236Arg) is responsible for Birk Barel mental retardation dysmorphism syndrome ;MIM 612292, a maternally transmitted developmental disorder (Barel et al. 2008 &Graham et al. 2016). We describe the clinical features in our patient, who has a mutation in the same gene, but different position (table 4-1). Graham et al. 2016 compared 15 patients, those found in members of the originally reported Arab-Israeli family and 4 patients recently recognized.

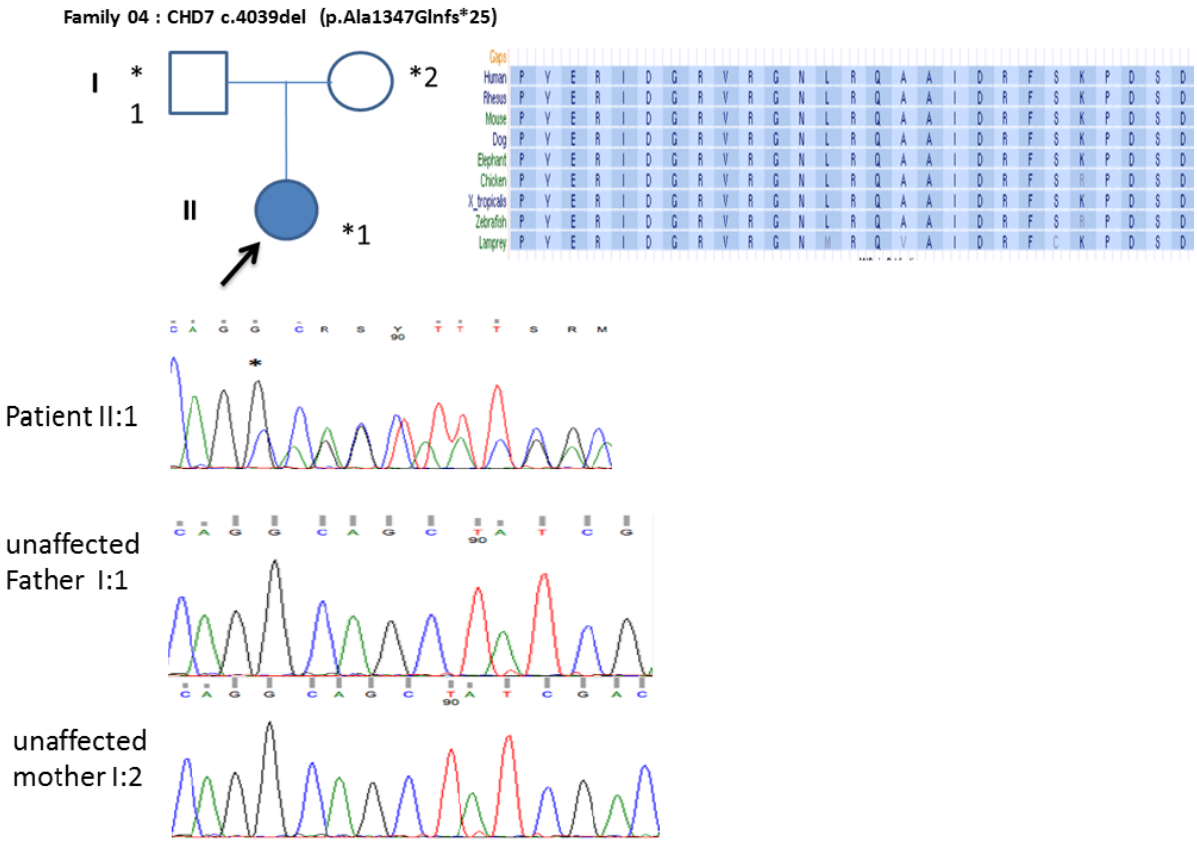


Figure 4-5: Family pedigree and transmission of De novo heterozygous mutations in the *CHD7* gene in family F04

Sanger sequencing analysis of the patient II:1 A heterozygous (1) bp deletion in the *CHD7* gene, c. 4039del, resulting in p. Ala1347Glnfs*25 l, was confirmed, heterozygous mutations are indicated by the asterisks (*). The Deletion replaces a highly conserved residue as shown in the multispecies up to lamprey (considering 9 species) and paralog alignment.

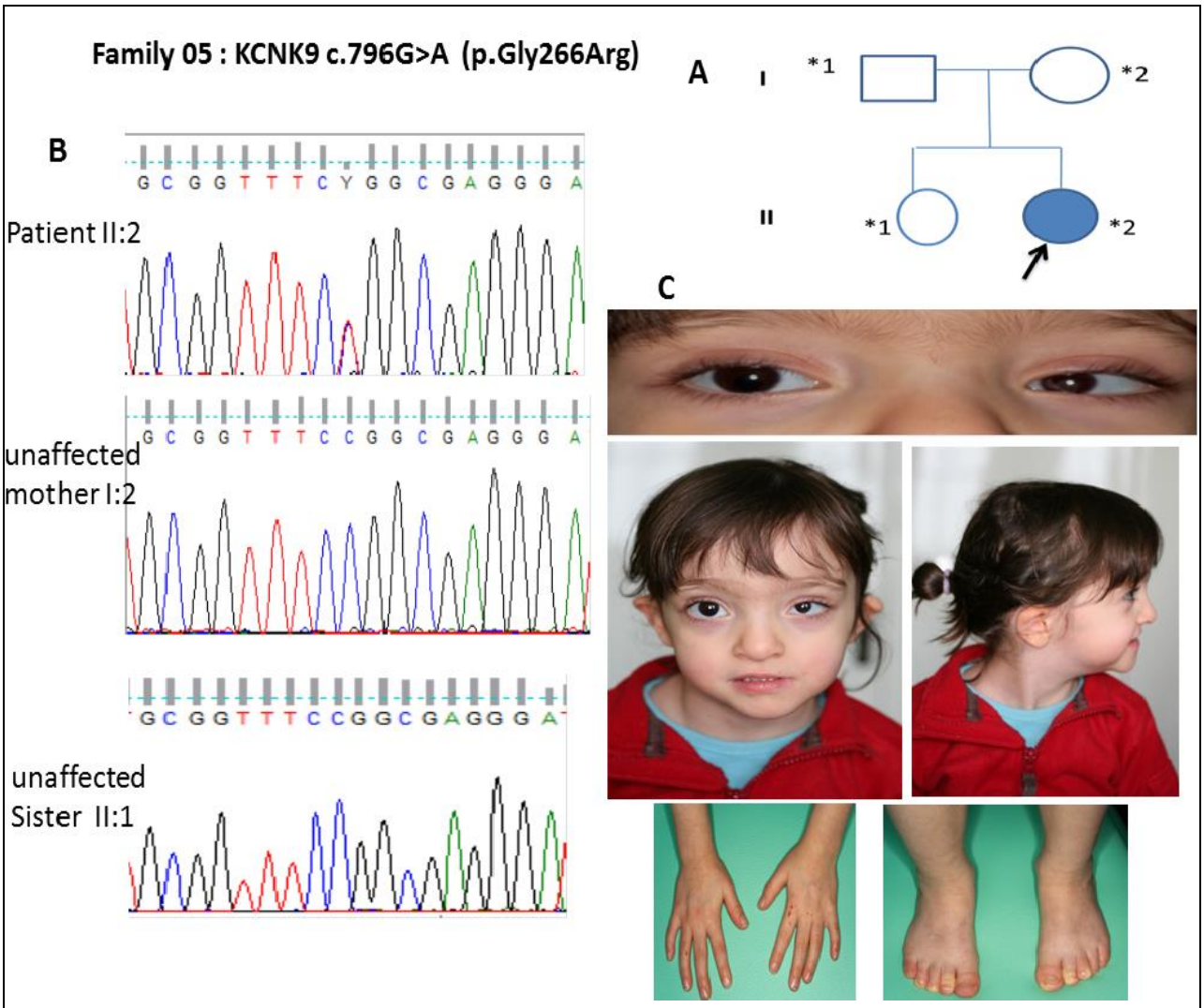


Figure 4-6: Family pedigree and Clinical features of the patients (II:2) in the family F05

(A) Family pedigree. (B) Sanger sequencing results of a healthy mother and her affected and healthy offspring. (C) Dysmorphic features with Cleft palate and eye defects (Coloboma / Microphthalmia) of affected sibling (see Table 4-1 for details)

FAMILY 06: Retinal atrophy/Microcornea/ Microphthalmia:

Before using NGS approach, the result of Array CGH test, confirmed no chromosomal abnormalities when the patient was 2 years old, and running a traditional Sanger sequencing analysis has confirmed that there is no mutation in *NIPBL* gene so exclude the Cornelia de Lange syndrome (CDLS; MIM 122470). Our filtering criteria were satisfied by two variations (*GDF3* and *ABCB6*), subsequent validation was carried on using Sanger sequencing to confirm and determine whether the candidate variant co-segregated with the phenotype within the pedigree. *GDF3* and *ABCB6* were inherited from a healthy father so we exclude these variatents , unfortunately until now we couldn't cover the causative variant, that with time phenotypes become appearance clear such as affecting most the ectodermal tissues comprising disturbance of skin pigmentation with verrucous patches and Blaschko lines, abnormal dental development, thick hair and eyebrows with Generalized hypertrichosis and nails, malformations of the eye, in addition to global developmental delay and microcephaly (see **Figure 4-7**).

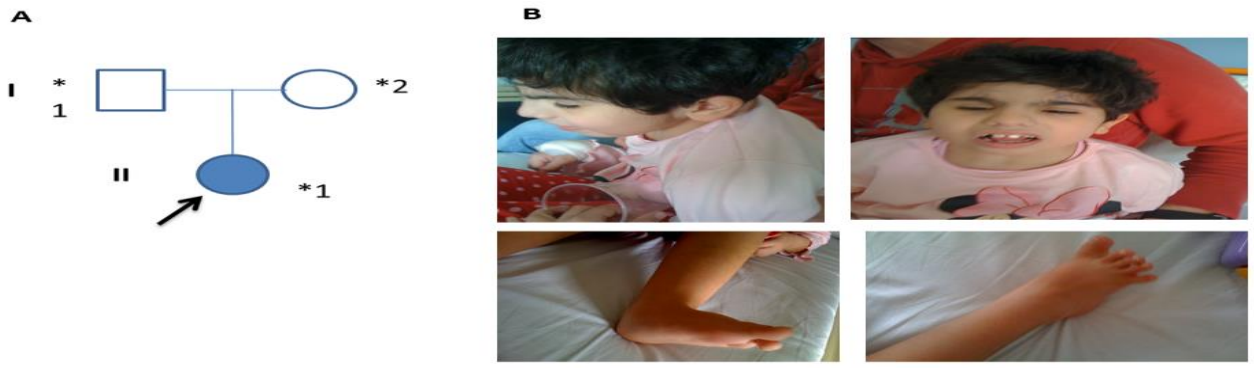
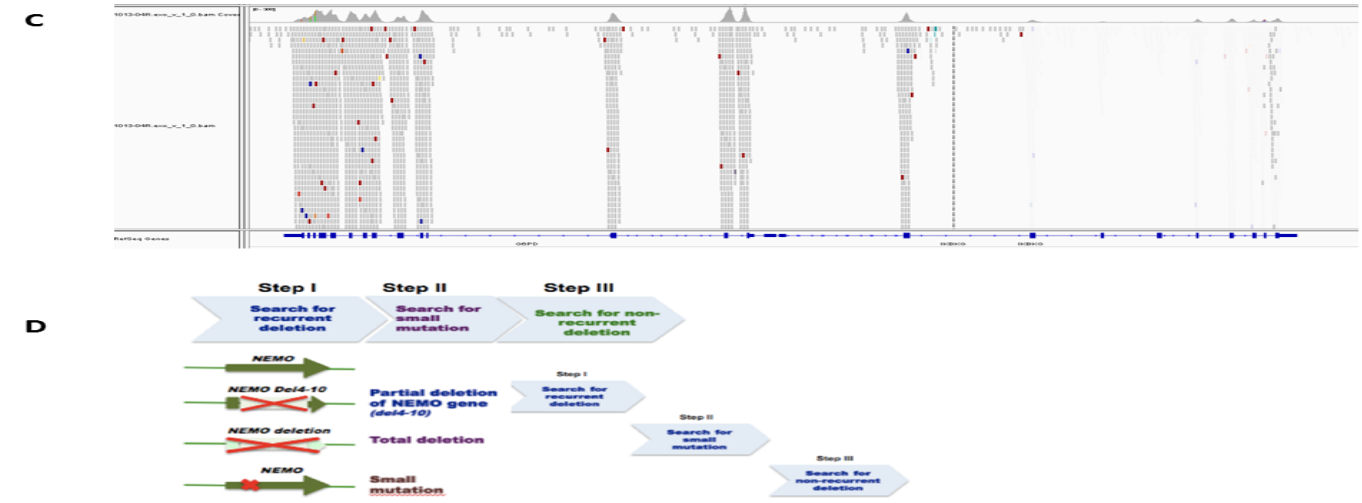


Figure 4-7: Family pedigree and Clinical features of the patients (II:1) in the family F06



(A) Family pedigree. (B) Photograph of the patient (II:1) of eye defect (Coloboma/Microcornea/ Microphthalmia) and other distinct phenotypes (see Table 3 for details). (C) A screenshot of the *IKBKG* gene window, as visualized by Integrative Genomics Viewer (IGV). Gray bars represent the mapped reads aligned to the reference genome, whose sequence is shown below. The coverage track is displayed in the sample. Regions of high sequence homology continue to be a major challenge for short-read technologies and can lead to false-positive and false-negative diagnostic errors (D) a three-step protocol for the molecular identification of *IKBKG* mutations in IP cohort Italy

4.2.2 Non-syndromic or Isolated inherited eye diseases

4.2.2.1 Autosomal Recessive

FAMILY 07: Leber congenital amaurosis 1 (*GUCY2D*):

In both affected brothers with Leber congenital amaurosis 1(LCA1;MIM 204000).We found homozygous deletion in exon 2 that creates a frameshift starting at codon Pro 130. The new reading frame ends in a STOP codon 35 positions downstream (c.387delC, p.Pro130Leufs*36) in GUCY2D gene, NM_000180.3. It represents a very rare variant (rs61749670) without frequency or population information. It is mentioned in one citation (Perrault et al.1996) and is therefore considered pathogenic by HGMD (CD962030). This variant was confirmed by Sanger in all affected family members (the patient and his brother), unaffected consanguineous parents were both heterozygous carriers for the mutation **(Figure 4-8)**

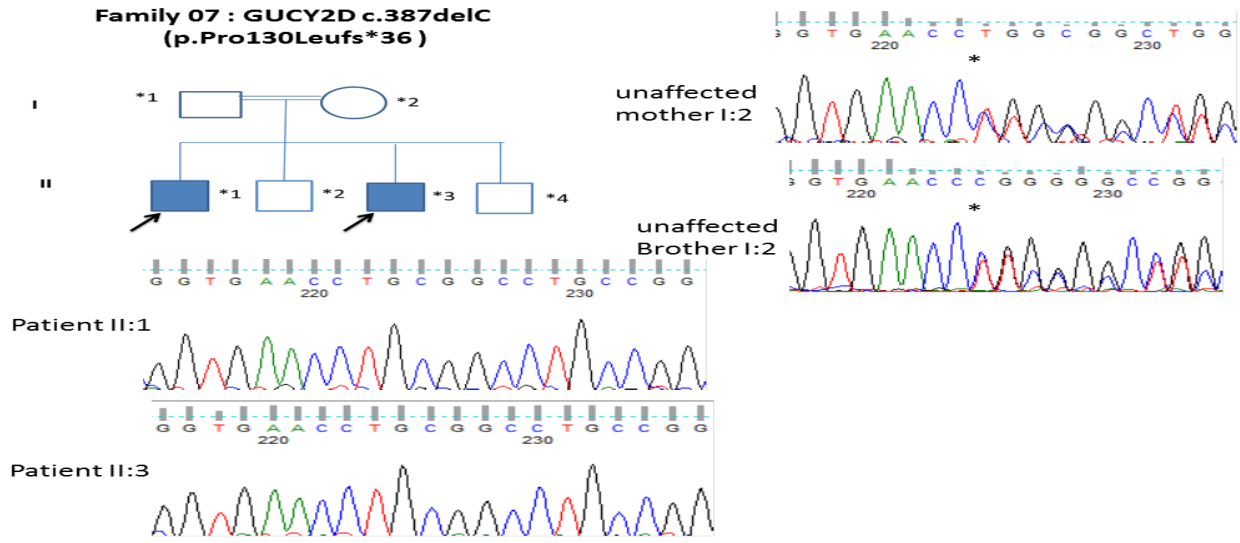


Figure 4-8: Family pedigree and transmission of homozygous mutations in the *GUCY2D* gene in family F07

Sanger sequencing analysis of the patients (II:1 and II:3) were confirmed A homozygous (1) bp deletion in the *GUCY2D* gene, c.387delC, resulting in (p.Pro130Leufs*36), heterozygous mutations are indicated by the asterisks (*) in carrier mother and her sibling.

FAMILY 08: Retinitis pigmentosa (*USH2A*):

We identified compound heterozygous missense mutation in *USH2A* (NM_206933.2: (c.1040A>G: p.Asp347Gly in exon 6 and c.10817T>C: p.Leu3606Pro in exon 55).They have already been reported in the literature (Sodi et al. 2014 & McGee et al. 2010, respectively), and therefore they are considered pathogenic by HGMD (CM1413399 and CM104143, respectively), and both could not be found in the 1000 Genomes Database (No dbSNP ID). They were labeled as deleterious by the predictive software Alamut 2.7.2. The mutations were confirmed by Sanger and the segregation with c.1040A>G: p.Asp347Gly from the father, and since the mother of the patient is dead, we couldn't investigate the mother's side of this screening, but it can be assumed that she is also a carrier for the second allele (c.10817T>C: p.Leu3606Pro)(**Figure 4-9**). They are predicted to be deleterious (SIFT), probably damaging (Polyphen2 score = 1.0) and disease-causing (MutationTaster2). Mutations within *USH2A* gene have been associated with autosomal recessive Retinitis Pigmentosa without hearing loss, in Retinitis pigmentosa 39; MIM 613809 or Usher syndrome, type 2A; *USH2A*, MIM 276901. To date, more than 1050 *USH2A* variants have been detected, the majority are single-base variants (substitutions, missense/nonsense).

FAMILY 9: Retinitis pigmentosa (*TULP1*):

The ophthalmic examinations of the 2 affected proband in this familial cases were suggestive of RP. After exclusion of all known mutations in all genes involved in autosomal recessive retinitis pigmentosa.We identified compound heterozygous mutations in *TULP1* gene(NM_003322.4) and the gene co-segregated with disease in the their family for one missense mutations affecting the conserved C-terminal region and intronic variant in *TULP1* gene: c.797G>T (p.Gly266Val) and c.823-17G>C respectively .The mutations were confirmed by Sanger and the segregation with c.797G>T (p.Gly266Val from the father and variant in *TULP1* represents a rare variant (rs150480343) that was detected heterozygous in MAF < 0.01 (A) .On the other hand the second allele inherited from the mother c.823-17G>C and this intron variant (rs12215920) is a little bit

frequency in general population (MAF: 0.30 (G)) . We examined the possible impact of the p.Gly266Val substitution on the *TULP1* protein using the PolyPhen-2 algorithm, which suggested that the valine substitution at position 266 would probably be damaging. We examined the evolutionary conservation of amino acid Gly266 and nucleotide c.823-17G and found that Gly266 and c.823-17G are completely conserved in TULP1 orthologs (**figure 4-10**).To date, more than 60 pathogenic mutations have been reported in TULP1, with the majority in sporadic patients with autosomal recessive RP, rod-cone dystrophy and leber congenital amaurosis (LCA).

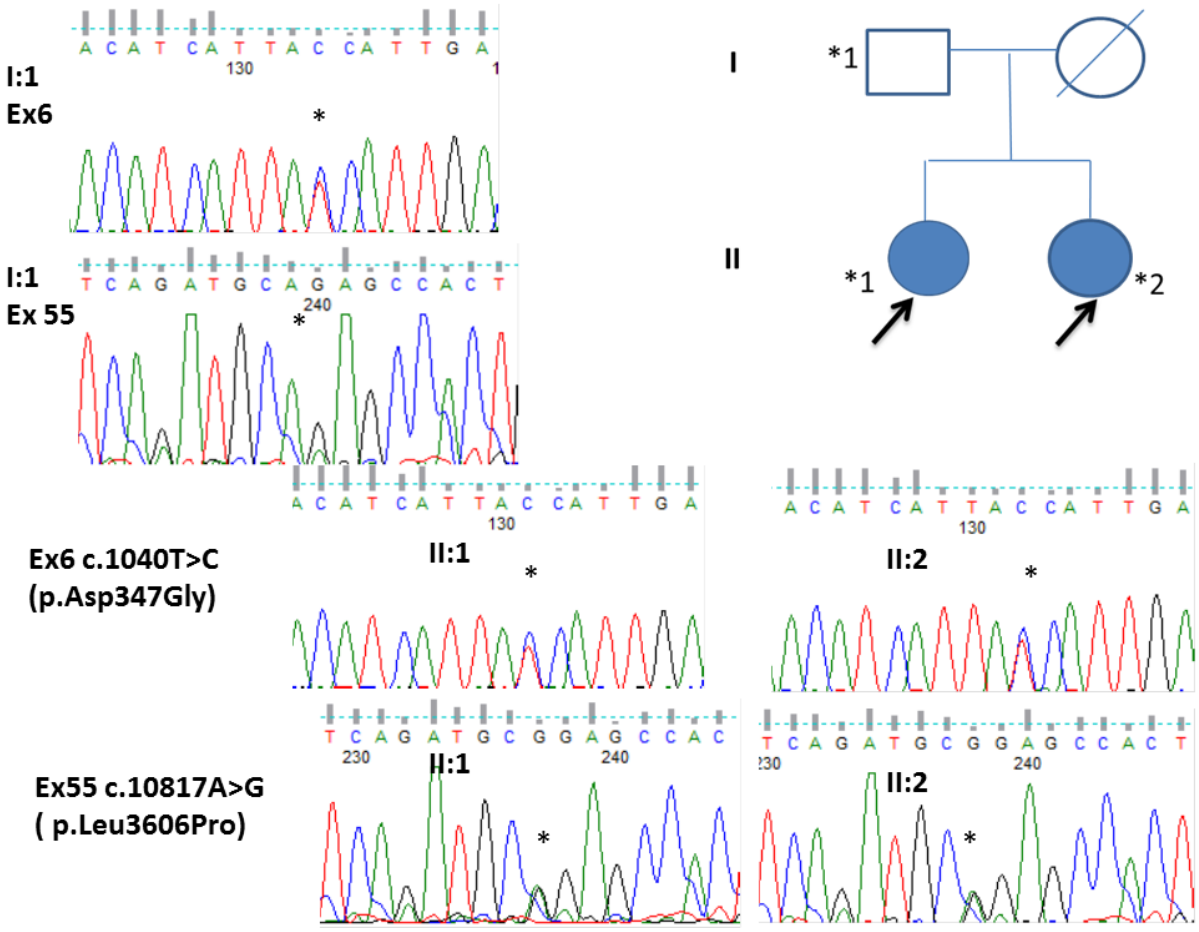


Figure 4-9: Family pedigree and transmission of the Compound homozygous mutations (c.1040T>C and c.10817A>G) in the *USH2A* gene in family F08

Sanger sequencing electropherograms for both loci are shown in patients (II:1 and II:2) in Ex6 and Ex 55 resulting in (p.Asp347Gly) and (p.Leu3606Pro) respectively. The mutations were confirmed by Sanger and the segregation with c. 1040A>G:p. Asp347Gly from the father, and since the mother of the patient is dead, we couldn't investigate the mother's side of this screen, but it can be assumed that she is also a carrier for the second allele (c.10817T>C: p.Leu3606Pro).

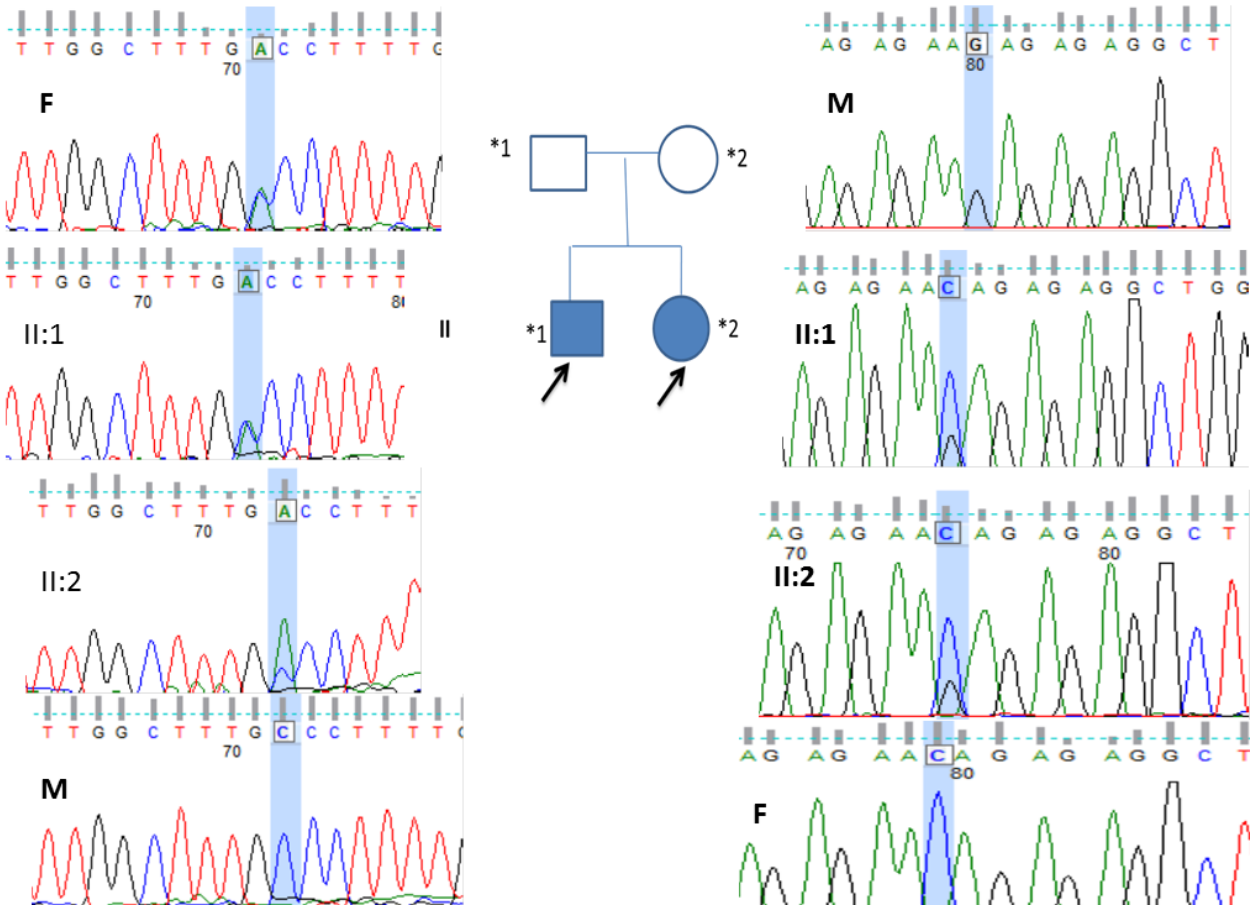


Figure 4-10: Family pedigree and transmission of the Compound homozygous mutations (c. 797C>A and c. 823-17G>C) in the *TULP1* gene in family F09

Sanger sequencing electropherograms for both loci are shown in the Left panel: Exon 8 region missense mutation: c.797G>T (p.Gly266Val) The sequences for both the Father (F) and his siblings II:1; II:2 show heterozygosity with C and A at the same location, while the mother's sequence is normal (M). Right panel: int 8 region missense mutation: c. 823-17G>C. The sequences for both the mother (M) and her siblings II: 1; II: 2 show heterozygosity with G and C at the same location while the fathers sequence is normal.

Family F10 : Stargardt Macular Dystrophy (*ABCA4*):

Total variant analysis of the two patient's WES data revealed 6816978 variants (single nucleotide variants and insertion/deletion (SNVs/InDels). After filtering against controls (our in-house database (n=107), 1000 genomes, ESP, with 1% of a population frequency and Stargardt macular dystrophy panel genes (*ABCA4 ,BEST1, C1QTNF5, ELOVL4, FSCN2, IMPG1, PRPH2,RDH12,RP1L1,RPGR* and *TIMP3*) or against eye defect gene list and using the subsequent manual inspection using the IGV browser .One potential candidate variant remained .Which represents a transition from G to A in exon 36 of the *ABCA4* gene, (NM_000350.2:c.5087G>A), leading to change from Ser at position 1696 to Asn (p.Ser1696Asn) it is Highly conserved amino acid, up to Frog .This variant is reported as pathogenic by Retino Genetics database (RetinalMD_00069) and HGMD-phenotype is stargardt disease (CM990061)(Lewis et al. 1999) and in Sanger sequencing of the specific ABCA4 region confirmed the presence of the variant identified by WES in the patient and excluded its presence in unaffected father, but confirmed its presence in unaffected mother (see **Figure 4-11**).And other two variants (*GPR98* and *CNGB3*), which after filtering out the data they were synonymous, with a population frequency above 1%, already found in the previous sequencing analysis of uncorrelated samples or intronic variant distant more than 20bp. Sanger sequencing conducted in the patients and in the father patients, revealed the variants (*GPR98* and *CNGB3*), No copy number variation (deletions and insertions) was detected in our patients in the ABCA4 locus.

4.2.2.2 Autosomal Dominant

FAMILY 11: Rieger or Axenfeld anomalies (*FOXC1*):

In correspondence with filtering approaches, one potential candidate variant remained which is a duplication (1bp) in in inhibitory domain of the coding region of the *FOXC1* gene, (NM_001453.2:c.780dup: p.Asp261Argfs*45) , which leads to frameshift starting at codon

Asp261, and results in e new reading frame that ends in a STOP codon 44 positions downstream.This variant is classified as pathogenic by HGMD as Anterior segment dysgenesis (CI117370) and was previously described (D'haene et al. 2011), and based on mutationtaster the resulted change in amino acid sequence (frameshift), and hence the truncated protein (might cause NMD). Sanger sequencing of the specific *FOXC1* region, confirmed the presence of the variant identified by WES in the patient and excluded its presence in unaffected mother, but confirmed its presence in affected father with aniridia (see **Figure 4-12)** Besides, by using a novel software package called EXCAVATOR to detect copy number variants (CNVs) from WES data, there are no dosage anomalies in the four genetic loci for (Anterior segment dysgenesis) , which are situated on chromosome 4q25 (*PITX2*) ,6q25 (*FOXC1*),13q14 and 16q24 (genes remain unidentified) (**Figure 4-12)**

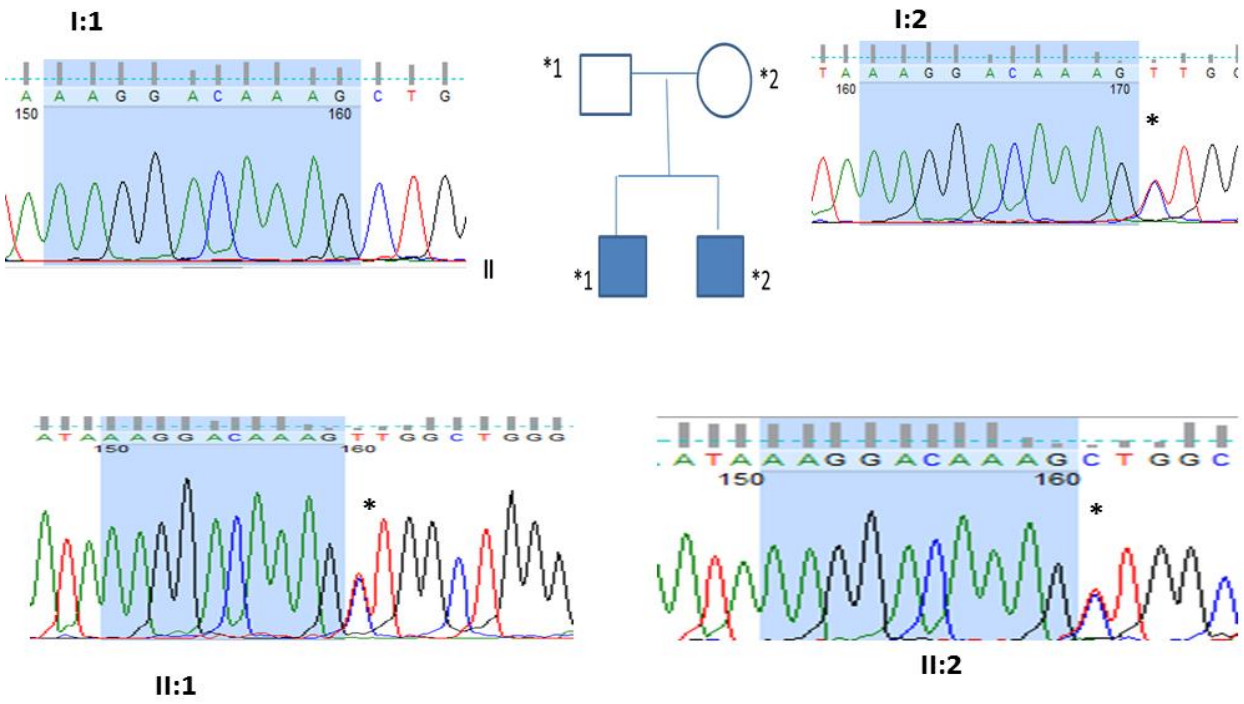


Figure 4-11: Family pedigree and transmission of the homozygous mutations (c. 5087G>A; p. Ser1696Asn), in the *ABCA4* gene in family F10

Sanger sequencing of the specific ABCA4 region confirmed the presence of the variant identified by WES in the patients (II:1 and II:2) and excluded its presence in unaffected father, but presence in unaffected mother, homozygous mutations are indicated by the asterisks (*) in carrier mother and her sibling.

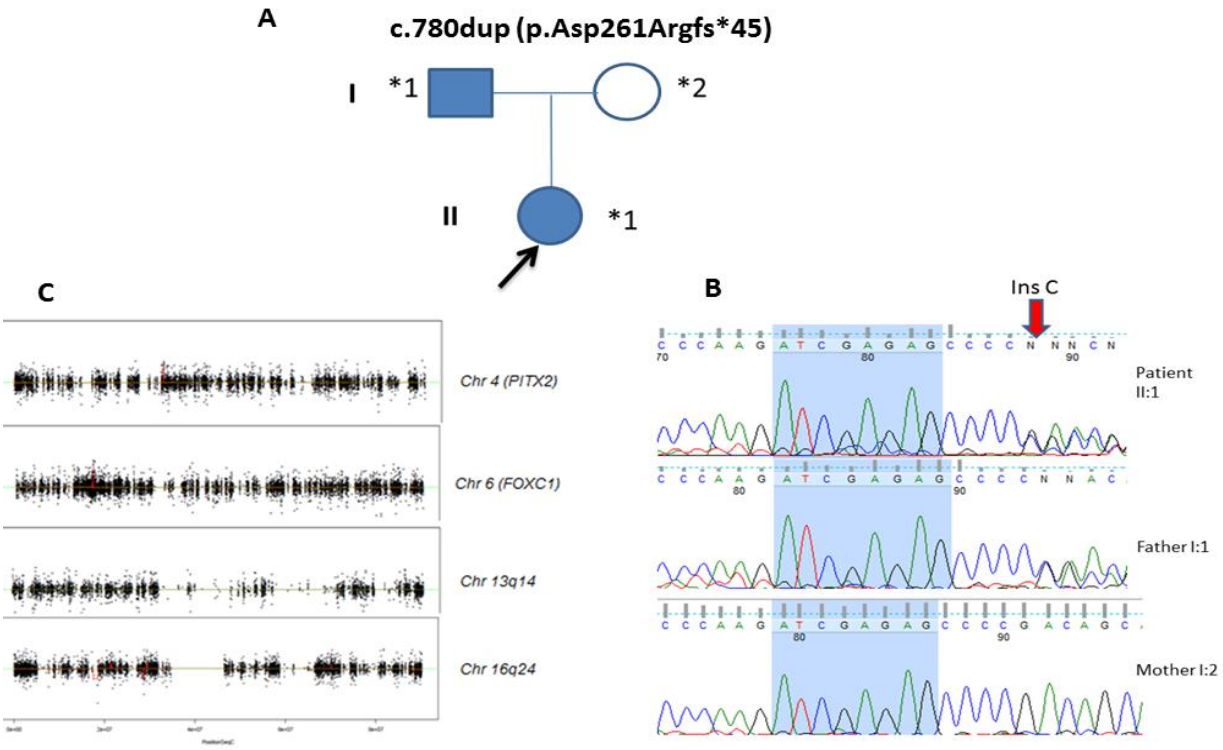


Figure 4-12

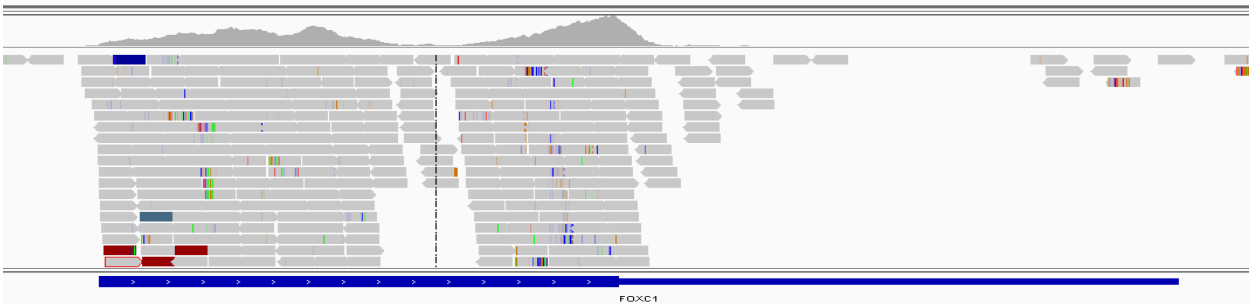


Figure 4-13: Family pedigree and transmission of the heterozygote mutations (c.780dup (p. Asp261Argfs*45) in the *FOXC1* gene in family F11

(A) Family pedigree. (B) Sanger sequencing analysis of the patient II:1 shows heterozygous (1) bp insertion in the *FOXC1* gene, c. 780dup, resulting in (p. Asp261Argfs*45), was confirmed in the effected father and his daughter II:1 and wild type in the healthy mother. (C) Copy number analysis by EXCAVATOR software shows No copy number changes in *FOXC1* and *PITX2* or in two known additional genetic loci (13q14, 16q24).

5 Discussion

NGS represents a great and useful tool to be used in genetic diagnostic assays, which strongly facilitates the classification of disease-related sequence variants in patients with genetic disorders. Its wide application has enabled the transition from a single gene and known mutation diagnostic assays, to full survey of variation within all candidates or known genes causing disease phenotypes. This has shifted the diagnostics for the detection of disease-causing variation in the clinical interpretation of an excess of rare genetic variants. We applied this technology to highlight causative coding variants in subjects with inherited, either syndromic or non-syndromic, eye diseases, representing a clinically and genetically heterogeneous set of Mendelian disorders that affect millions of individuals worldwide (Berger et al 2010), that were already mentioned in HGMD, LOVD, OMIM, ClinVar, Retinal Information Network, RetinoGenetics database and the most recently literature research. The aim of this study was to provide the families a prognosis and a genetic counseling in respect to the risk of recurrence of the disease in following pregnancies but also to better understand about rare variants and rare phenotypes and get novel genotype-phenotype relationships in rare diseases such as MAC spectrum of congenital ocular malformations (Microphthalmia/ Anophthalmia/ Coloboma),CHARGE syndrome, Axenfeld-Rieger syndrome and others given that WES can identify both exonic SNVs and small CNVs in disease-causing genes, those platforms can facilitate differential diagnosis, suitable therapy and genetic counseling for patients with these syndrome and related diseases in a cost-effective manner.

Here, we report three consanguineous and eight non-consanguineous families, that were recruited from Italy with multiple members manifesting a hereditary ocular disease. We identified four novel and previously reported variants, all segregating with the disease phenotype in the respective families.

Several cases in our cohort were the first affected person in their family, but in the same time there were concerns about recurrence possibility or opportunity a expressed by family members and the individuals themselves, thus identification of causative alleles responsible for inherited visual impairment will support diagnostic efforts to identify carrier status in intermarried familial cases, and following genetic counseling will aid families make rational decisions concerning arranged marriages and screening for the status of conceived baby or newborns.

5.1 Novel disease-causing variants explaining the phenotypes in our cohort

Family 01 (*RAB3GAP1*)

We reported a novel homozygous mutation in the catalytic subunit of RAB3GAP1 (c. 2874C>G:p.Tyr958*), in patient F01;IV:1(**Figure 4-2**). This gene encodes the catalytic subunit of a Rab GTPase activating protein. The encoded protein forms a heterodimer with a non-catalytic subunit to specifically regulate the activity of members of the Rab3 subfamily of small G proteins. Rab3 protein is complexed in regulated exocytosis of neurotransmitters and hormones. It might participate in neurodevelopmental processes such as proliferation, migration and differentiation before synapse formation, and non-synaptic vesicular release of neurotransmitters. Specifically, it converts Rab3-GTP (active) to the Rab3-GDP (inactive) form. It is required for normal eye and brain development.

The mutation was identified in a female Italian sibling presenting an extremely complex and rare genetic disorder with microcephaly, hypotonia and eye defects (microphthalmia, congenital cataract, microcornea, coloboma, optic nerve hypoplasia, aphakia). On the basis of previous reports, once viral infection and chromosome abnormalities have been excluded, there was a strong possibility, that she was affected, by a genetic syndrome such as Cerebro-oculo-facio-skeletal syndrome (COFS), Cockayne syndrome (CS), CAMFAK syndrome, MICRO syndrome, Martsolf syndrome, and CAHMR syndrome, all sharing some phenotypic abnormalities present in the child. (for more details see Morris-Rosendahl et al. 2012). Without WES it would have

been difficult to arrive to a molecular diagnosis. In fact the commercial panel for microphthalmia syndromes does not contain any of these syndromes. The most suitable solution was to scan the whole exome.

With the increased availability of molecular genetic diagnostic tools, made it crystal clear, that different clinical entities can be caused by mutations in the same gene, and that a clinically homogenous entity can be caused by mutations in different genes. So, WES, offers the possibility to map entire genomes at affordable costs to scan for any possible causative mutation gene, or to identify novel disease genes, which explains the resulted heterogeneous phenotype. Instead our WES approach allowed us to frame the disease within the Warburg Micro syndrome (WARBM1; MIM 600118), caused by a novel mutations in *RAB3GAP1*. The child is now under standard supportive therapies for developmental delay.

Family 03 (*CDH23*)

The affected male sibling (F03; II: 1), shows congenital deafness and a high degree of retinal degeneration. We identified bi-allelic missense mutations on the Cadherin protein domain of the *CDH23* gene, a gene associated with Usher syndrome type I (**Figure 4-4**). The first allelic mutation (c.4027C>T: p.Leu1343Phe) was de novo and not reported in any database or literature. All the prediction tools consider it as disease causing. The second allelic mutation (c.3134G>T: p.Cys1045Phe), is reported as an SNP with an extremely rare variant with T: 0.001% frequency according to ExAC. However, the large physicochemical differences between Cys and Phe (Grantham Dist.: 205 [0-215]) and Align GVGD: C0 (GV: 245.67 - GD: 0.00) makes it a good candidate for being causative together with the first mutation.

Usher syndrome type I is currently thought to be associated with at least six genes, which are encoded by over 180 exons (*MYO7A, USH1C, CDH23, PCDH15, USH1G,* and *CIB2*) (Kinga et al. 2014). Thus, although we could exclude the involvement of other variants in these genes, we

are not sure that the (c.3134G>T: p.Cys1045Phe) variant is indeed associated with the disease of our child. Of course, only functional data might demonstrate its real role.

Family 04 (*CHD7*)

Here, we report a novel heterozygous frameshift mutation of *CDH7* (c.4039del G: p.Ala1347 Glnfs*25), in an Italian patient with CHARGE syndrome ((**Figure 4-5**). Clinical diagnosis of CHARGE syndrome may be difficult, because other diseases share its clinical features. Moreover, the *CHD7* gene has numerous exons, (38 exons covering 188 kb a genomic size) (Kohmoto et al.2016). For that reason, NGS is the most accurate and definitive method for differential diagnosis. In our case the finding of a de novo 1bp deletion in exon 17 made very straightforward the role of this variant in the disease phenotype although the variant is a novel one.

In fact, according to HGMD professional 2016.2, there are 738 *CHD7* mutations in CHARGE syndrome. The majority of these mutations are nonsense or frameshift as in our case; indeed suggesting that haploinsufficiency for *CHD7* is the most common pathogenic mechanism underlying CHARGE syndrome. We may hypothesize that our variant produces an aberrant mRNA that will be targeted for degradation via nonsense mediated decay (NMD). No other possible pathogenic SNVs and CNVs were detected in *CHD7* or in other genes associated with the phenotype of our child.

Family 05 (*KCNK9*)

The mutation, a novel one, is in the topological domain of TASK3 channels (*TASK-3 /KCNK9* : c.796G>A, p.Gly266Arg).in (F05;II:2) patient , TASK3 channels are highly sensitive to changes in extracellular pH . The clear differential expression of this gene in the brain, where only the maternal allele is expressed (Peters et al, 2014), classifies it as a new imprinted gene at 8q24.3 that adds to the about 70 already known.

In the literature and HGMD professional, the Birk Barel mental retardation, dysmorphism syndrome is caused by the same mutation, p.Gly236Arg; rs121908332, affecting the *KCNK9/TASK3* gene detected in around only 19 cases from five unrelated families (Barel et al. 2008 &Graham et al. 2016). It has been demonstrated that the dysfunctional TASK3 channel. impairs normal migration of cortical pyramidal neurons during embryogenesis.

We report here an extremely rare variation (rs370302731;T: 0.001%) in *KCNK9* mutations, in an Italian female sibling presenting a complex genetic disorder and a phenotype dominated by intellectual disability, hypotonia , feeding difficulties in infancy , unique facial dysmorphism with cleft palate and eye defects (Coloboma / Microphthalmia) **(Figure 4-6, Table 4-1)** and thus largely overlapping with the phenotype of the Birk-Barel syndrome. The already described variant falls in a transmembrane domain and resulted to be causative when maternally inherited. In our case, the variant is in the contiguous domain, a topological one, and resulted to be de novo. It seems likely that it was inherited by the mother, thus resulting in the expression of an abnormal protein and the silencing of the normal gene. Studies to deep this point are in progress. Once again NGS was pivotal in the detection of the causative variant, and allowed us to identify an interesting and unexpected phenotype-genotype relation after the exclusion of any chromosomal abnormalities or any mutation in Coloboma / Microphthalmia genes panel.

Family 09 (*TULP1*)

We identified bi-allelic mutations in *TULP1* (c.797G>T :p.Gly266Val) and (c.823-17G>C,) in the two affected siblings with severe, early-onset retinitis pigmentosa (see **figure 4-10**). Tulp1 is expressed exclusively in photoreceptors (retina) that use ribbon synapses to communicate with the inner retina. Mutations in *TULP1* are found in 1-2% of arRP cases in different ethnic populations worldwide (Ullah et al. 2016). Tulp1 belongs to the tubby family of proteins (Tulp2, and Tulp3). All 3 genes encode proteins with different N termini but with highly conserved

carboxyl terminal "tubby" domain of ~250 amino acids, which binds to phosphoinositol-4,5-bisphosphate (PIP_2).

To date, according to HGMD professional 2016.2, more than 60 pathogenic mutations have been reported in *TULP1*, with most of them in sporadic patients affected by autosomal recessive RP-14 (MIM 600132), Leber congenital amaurosis LCA-15 (MIM 613843 or rod-cone dystrophy. It is difficult to demonstrate that intronic variant c.823-17G>C is indeed pathogenic without transcript analysis investigation in patient fibroblasts, although we may hypothesize that this variation affects the splicing of *TULP1* by activating a cryptic splice-acceptor site.

5.2 Recurrent disease-causing variants

Family 02 (*STRA6*)

We reported homozygous mutations in *STRA6*, an important regulator of vitamin A and retinoic acid metabolism (c. 1964G>A: p. Arg655His), that previously classified as pathogenic in two siblings with microphthalmia syndromic 9 (**Figure 4-3**). Our patients have somehow a milder phenotype than the previously reported patients, with remnant eye globes and intact optic nerves in F02; II: 2. The older sister (F02; II: 1) has hypoplasia of the optic nerve, as well as pulmonary atresia with ventricular septal defect *(*PA-VSD*)* and right aortic arch. Both sisters show a normal psychomotor development, no renal disease, and no diaphragmatic hernia. To date, no more than 50 different mutations of *STRA6* have been reported in the literature and HGMD, professional 2016.2. Our data strongly support the results of other authors, indicating that the phenotypic spectrum of *STRA6* mutations might be broader with expression variability higher than originally suspectedleading to either very severe conditions or isolated micro/anophthalmia (Segel et al. 2009 & Marcadier et al. 2016). *STRA6* analysis should be considered in all patients with MAC spectrum and genetic counseling should be cautious with respect to long-term outcomes.

Family 07 (*GUCY2D*)

It is interesting to note that the two affected siblings in F07 have a single bp homozygous deletion in the extracellular C-terminal domain of *GUCY2D* (c. 387delC: p.Pro130Leufs*36), **(Figure 4-8).** The deletion creates a stop codon at 166 positions of the cDNA, which results in a larger, most likely unstable, mRNA or a dysfunctional protein and confirm the primary diagnosis of the patients of Leber congenital amaurosis 1. Our results are consistent with those reported by Perrault et al. 1996 who found the same variant in a patient with a totally overlapping phenotype. *GUCY2D* is a well conserved protein, containing an extracellular receptor domain, a transmembrane domain, an intracellular protein kinase homology domain, and an intracellular guanylate cyclase domain. 49 mutations have been so far described in all these domains. An altered *GUCY2D* protein could indirectly disturb the regulation of phototransduction in the photoreceptor cells, leading to RP or cone-rod dystrophy 6, as well as in LCA with noteworthy phenotypic overlap (Booij et al. 2005).

Identification of causative alleles responsible for such type of autosomal recessive diseases, will aid to identify carrier status in intermarried familial cases, and provide proper genetic counseling, in making rational decisions regarding arranged marriages and screening for the status of newborns. Indeed, this was the case of family F07, where the parent asked for a prenatal diagnostic test for their fourth child to scan for *GUCY2D* mutation.

Family 08 (*USH2A*)

We identified bi-allelic mutations in the long isoform of the *USH2A* gene in patients with Usher syndrome type II or non-syndromic retinitis pigmentosa (**Figure 4-9**). *USH2A* is the most frequently gene involved in Usher syndrome type II (USH2) and the prevalence of the disease has been estimated at about 3.5-8: 100,000 in European populations (McGee et al. 2010; Sodi et al. 2014& Ellingford et al. 2016).

The first allelic mutation (c.1040A>G: p.Asp347Gly) was in the Italian patient diagnosed with Usher syndrome 2, but carries another second hit that we identified. Also the second allelic mutation (c.10817T>C: p.Leu3606Pro) in our patients was in the one who was diagnosed with Retinitis pigmentosa. These results demonstrate the great potential of the WES strategy that we adopted in our investigation in RP or Inherited retinal degeneration diagnosis as a general. Better knowledge of molecular alterations underlying RP in specific populations may lead to more efficient diagnostic strategies and future therapeutic approaches.

Family 11 (*FOXC1*)

In our cohort, F11, II: 1; (**figure 4-12**), is an isolated case with autosomal-dominant Axenfeld-Rieger anomaly, a severe disorder affecting vision characterized by extreme genetically heterogeneity. We have identified a one bp insertion in *FOXC1*, it is a member of a gene family that is characterized by an evolutionarily conserved 110 amino acid, DNA-binding forkhead domain (FHD). We identified mutations downstream of the FHD in inhibitory domains ID (p.Asp261Argfs*45).The same mutation had been previously reported in a family showing Anterior Segment Dysgenesis (ASD). Unfortunately, no further ocular details are provided (D'haene et al. 2011). According to HGMD, professional 2016.2, there are no more than 120 different mutations reported in *FOXC1*. Th e more common ones are loss-of-function mutations associated with Axenfeld-Rieger syndrome (Smith et al. 2000).

In case of ASD, it is important to examine the patient annually because of the risk to develop juvenile glaucoma. In fact, in the father's branch of our patient, two persons suffer from juvenile glaucoma, suggesting that they may carry the same causative variant although not affected by ASD. As the mutation spectrum for both *PITX2 and FOXC1*, both associated with Axenfeld-Rieger anomaly, is very broad, several different methods should be applied for the genetic diagnosis of ASD. But by using WES, we can easily scan for intragenic variants, or CNVs such as deletions /duplications discovered by using the EXCAVATOR tool, A causative CNV was in

fact reported the literature It's important to keep in mind that, in this case we had to implement our WES analysis by Sanger sequencing of the entire region that was with low coverage (chr6:1,611400-1,612180).

5.3 Individuals without molecular diagnosis

In two families (one potentially of a recessive case, and one with a putative dominant mode of inheritance), we were not able to identify the variation in genes presently associated with inherited (developmental) eye diseases in human or animal models, or explain other phenotypic defect. These cases are being further investigated for identification of novel disease associated gene mutations.

Family 07 (*NEMO*)

In further investigations, we couldn't identify any candidate variant which might help us to understand this complex syndrome.There are several possibilities for not being able to identify the genetic cause by WES. In fact, the causative variant may be outside the coding sequences or a large indel variant on involving exons. ,Another important cause relies on a lower sequence coverage of the variant. In this family, after comprehensive meeting with the clinicians, we pointed out may the suspicion of Incontinentia pigmenti (IP; MIM #308300) an X-linked dominant disease , typically sporadic, caused by mutation in the IKK gamma gene (*KBKG/NEMO* NM 003639.3) in more than 80% pf the IP patients. , affecting the skin in all patients also the severity of the disease is correlated to neurological and/or ocular impairment (Ophthalmologic are present in approximately 20%-37% of IP patients) (Fusco et al. 2012 & Conte et al. 2014). Unfortunately, *NEMO* gene is not well covered by any of the commercial targeted enrichments kits used for WES. (see **Figure 4-7**) This is a good example showing that WES may not be enough to scan the possible causative variant which *IKBKG*. IP locus considered a genomic instability region because structural architecture where the gene is located (partially overlaps with *G6PD*, NM 000402 and with highly repetitive sequence, In addition, in

the same locus, there is a nonfunctional and highly conserved pseudogene copy (*IKBKGP*) (see **Figure 4-7**). As the consequence,recently we have contacted a number of professional centers involved in molecular diagnosis of IP in Italy, I.P.ASS.I. Onlus (www.incontinentiapigmenti.it), in order to perform an IP molecular diagnosis. Their strategy consists in a three-step procedure for the molecular detection of *IKBKG* mutation: (1) recurrent deletion, (2) small exon mutations (indel), and (3) nonrecurrent deletions, at the IP locus (see **Figure 4-7**). Until now we exclude 4-10 exon deletion. Although a recurrent deletion (IKBKGdel), is frequently associated with IP (78% of cases), the molecular diagnosis is quite complicated because of the *IKBKGP/NEMOP* and due to variations are scattered all along the gene (Fusco et al. 2012).

Family 10 (*ABCA4)*

We identified one pathogenic mutation in *ABCA4* (NM_000350.2: c. 5087G>A; p. Ser1696Asn). The *ABCA4* gene encodes a transmembrane protein is localized exclusively at the rims of the outer segments of cone and rod photoreceptors, so the absence of a functional assay for ABCA4, it is difficult to establish a role in pathogenicity for any individual *ABCA4* variant, particularly missense substitutions. But, the absence of the variation in a large control population, their confirmed co-segregated with the disease, and there are in conserved functional domains for many of the mutant alleles indicate that these mutations cause disease and are not benign and this is compatible with the variant in the two patients with simplex Stargardt Macular Degeneration (STGD) (MIM 248200). According to literatures, there are many explanations that interpret how one pathogenic variant in *ABCA4* which could be the causative mutation, although is the recessive form.

a. Digenic inheritance has been previously reported in retinal degeneration and in patients with SD (Maugeri et al.2002; Cooper et al. 2013& Lee et al. 2014) . Lee and his college have been suggested according to preliminary analysis that double carrier status for *ABCA4* and other retinal degeneration disease gene is rare in the general population,

supporting the possibility that dual heterozygous mutations in two STGD genes could be causative for STGD (Lee et al. ,2014). Our patients harbor one pathogenic variant in *ABCA4* inherited from maternal and one pathogenic variant in a second retinal disease gene (*GPR98* or *CNGB3* see **table 4-2)** inherited from paternal, so digenic inheritance may offer an explanation for a subset of these patients.

b. Deep intronic variants in *ABCA4*: Numerous reports have confirmed that approximately 15% of patients with a clinical diagnosis of Stargardt disease have only one identifiable mutation in the coding region of *ABCA4* (Chiang JP & Trzupek K.,2015).There are recent studies designed to find the missing disease-causing ABCA4 variation such as Braun et al. 2013, identified five deep intronic mutations in *ABCA4*. In the second report ,114 patients with only one already known *ABCA4* mutation were studied. Deep intronic mutations were identified in an additional (23.7%); no second mutations were identified in (31.6%) and intronic variants of unknown significance were identified in the remaining (44.7%) patients (Zernant et al. 2014). However, the prediction and confirmation of pathogenicity for the other intronic variants has proven difficult, mainly when the variant is rare. The detection of rare deep intronic variants in the ABCA4 gene, which is a highly polymorphic gene and not uncommon (Chiang JP & Trzupek K.,2015)

c. Incomplete or reduced penetrance has also been defined for autosomal recessive disorders in the retinal degeneration (Maugeri et al. 2002 & Cooper et al. 2013).

d. Spectrum of *ABCA4*-related phenotypes. Decreasing function of ABCA4 is thought to be linked with age-related macular degeneration (AMD) in some mutation heterozygous. Stargardt (STGD) patients have at least one missense allele; in cone-rod dystrophy (CRD) and retinitis pigmentosa (RP) patients have two severe mutations.

5.4 NGS limitations

WES is an effective method to identify disease-causing mutations, mainly from exceptionally heterogeneous disease with clinical heterogeneity and genetic heterogeneity. It is important to be aware of the NGS limitations (Huang et al. 2015); WES fails to identify disease-causing mutations in almost 20% of the cases. The possible reasons include:

1) The lack of sequence coverage of the variant, may impair the detection of causative variants. This condition occurs essentially in highly repetitive and GC-rich regions.For example, regarding the *RPGR* gene (NM_001034853.1), only 70% of the whole gene was covered by at least 20X, Because of purine-rich regions and the highly repetitive sequences in open reading frame 15 (*ORF15*) that is the last exon of the *RPGR* gene in which nearly 60% of disease-causing. However, the rest of the gene was greatly covered. To date, other alternatives have been effectively achieved to sequence *ORF15* in those cases in which attempts at characterization by NGS had failed (Huang et al 2015); Therefore, regions of high sequence homology continue to be a major challenge and can lead to false-positive and false-negative variant calls.

2) Variations in genes not yet disease-associate.

3) The causative variant of the disease is located outside the coding sequences that lie within deep intronic regions and are therefore missed by the exome capture methods.

4) Limitations of the employed method that prevent efficient identification of sequence alterations. Complementary methods, for example autozygosity mapping or WGS may be considered to facilitate the identification of the disease-associated genetic alterations.

5) Bioinformatics variant calling issues, and misinterpretation of variants.

6) Clinical heterogeneity or incorrect diagnosis may have falsely impacted our filtering strategy.

6 Conclusions and perspectives

In summary, we show the powerful clinical utility of a diagnostic NGS test for individuals with genetic visual impairment. With this methodology, we were able to get a molecular diagnosis in 13 of the 16 probands.

In 12 cases the causative variant was in genes already known to be associated with a visual impairment either isolated or syndromic. In four of them the variant was a novel one but either its segregation with the disease phenotype in the respective families or the prediction algorithms indeed did indicate its causative effect. Variants in genes not previously associated with eye defects were detected in (F05;II:2) case. It is interesting to note that in families F03 and F09 we imputed as causative a variant that is a SNP having a very low frequency. The vast majority of disease-linked SNPs has yet unidentified pathophysiological relevance. While cis-acting SNPs have been hypothesized, and more recently partly demonstrated, to either increase or decrease the final mRNA product (McClellan J. & King, MC, 2010; Soldner et al, 2016), those within the coding sequence may result in a bit less effective protein, that is disease-causing in presence of loss-of-function variant in the second allele as is the case in our patien F01,IV:I; F04,II:1; F07,II:1&II:2 or due to the silencing of the second allele for an imprinting condition, as is the case for our patient F05;II:2

In conclusion, our diagnostic approach resulted to be efficient, fast, and reliable. Although more expensive, the exome approach could be more successful than NGS dedicated panels that would have failed in at least three cases. This point has been clearly highlighted in several other instances (Shanks et al. 2013; Glöckle et al. 20014; Tiwari et al, 2016b & Sundaramurthy et al. 2016).

Finally, this study further remarks the noteworthy genetic and phenotypic heterogeneity underlying developmental eye defects and the challenges associated with genetic testing.

The promising potentials and the diverse applications of NGS, indicate that this technology will soon become the first tier test in clinical laboratories, strongly improving genetic counseling and, last but not least, contributing to novel therapeutic trials that require a prior knowledge of the mutation type

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List of original manuscripts

- "Whole exome sequencing identifies a novel homozygous mutation in the RAB3GAP1 and STRA6 genes as a causative variant in patients with sporadic complex ocular phenotypes involving Microphthalmia syndrome" to submit manuscripts.

- "SMARCA4 inactivating mutations cause concomitant Coffin-Siris syndrome, microphthalmia and small cell carcinoma of the ovary, hypercalcemic type" to submit manuscripts.