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(54) Title: METHOD FOR DIAGNOSING OCULAR ANTERIOR CHAMBER DYSGENESIS

(57) Abstract: The present invention relates to a method for diagnosing ocular anterior chamber dysgenesis.



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Method for diagnosing ocular anterior chamber dysgenesis

FIELD OF THE INVENTION

5 The present invention relates to a method for diagnosing ocular anterior chamber dysgenesis.

BACKGROUND OF THE INVENTION

10 Inherited congenital microcoria (MCOR) [MIM156600], also referred to as congenital miosis, is a rare inborn error of iris development. This disorder is a type of ocular anterior chamber dysgenesis since the pupil and iris anomalies are associated with goniodysgenesis (prominent iris processes and high iris root insertion) and glaucoma. Ocular anterior chamber dysgenesis in which abnormal cleavage of the ocular anterior chamber occurs, predispose to the development of glaucoma. MCOR is characterized by a small pupil (diameter < 2 mm) that dilates poorly or not at all in response to topically administered mydriatic drugs. Dilation inability results from absent or incompletely developed
15 dilator pupillae muscle. The sphincter pupillae muscle which acts in opposition to the dilator muscle to cause constriction of the pupil is unaltered. In addition to abnormal dilator pupillae muscle, the miotic iris is thin and it displays abnormal stroma and iridocorneal angle.¹⁻⁴ Iris thinning is consistent with transillumination of miotic irises and high sensitivity to light. High myopia and glaucoma are frequently associated with this condition.⁵⁻⁸

20 MCOR is a bilateral disease transmitted as an autosomal dominant trait with complete penetrance. A unique 8 Mb locus on chromosome 13q31-q32 has been mapped in 1998 by linkage analysis⁹ in a large multigenerational French pedigree first described in 1964.¹⁰ Gene mapping in some other families confirmed linkage to this locus¹¹ whereas some others were inconsistent with the 13q31-q32 region, supporting genetic heterogeneity of the disease.¹²

25 At present, there is no method for early diagnosing of ocular anterior chamber dysgenesis and for predicting the risk of developing disorders resulting from ocular anterior chamber dysgenesis such as inherited congenital microcoria (MCOR) or glaucoma.

30 SUMMARY AND DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method for diagnosing ocular anterior chamber dysgenesis or predicting the risk of developing a disorder resulting from ocular anterior chamber dysgenesis, in a subject, said method comprising detecting a defective *GPR180* gene encoding the G protein-coupled
35 receptor 180 in a sample obtained from said subject, wherein the presence of a defective *GPR180* gene is indicative of ocular anterior chamber dysgenesis or of a risk of developing ocular anterior chamber dysgenesis.

The present invention also relates to a method for predicting a risk of a subject to transmit a disorder resulting from ocular anterior chamber dysgenesis to offspring, said method comprising detecting a defective *GPR180* gene in a sample obtained from said subject, wherein the presence of a defective *GPR180* gene is indicative of a risk of transmitting a disorder resulting from ocular anterior chamber dysgenesis.

Indeed, the inventors have shown that defective *GPR180* gene is involved in the pathological process of ocular anterior chamber dysgenesis and of disorders resulting from ocular anterior chamber dysgenesis such as inherited congenital microcoria (MCOR) or glaucoma.

Typically, the subject according to the invention is a mammal, preferably a human.

The subject may be an adult, a teenager, a child, an infant, a baby or a foetus.

Typically, in the case where the subject is a foetus, the sample may be an amniocentesis sample.

The sample may be any biological sample derived from the subject which may contain DNA, RNA or the G protein-coupled receptor 180. Examples of such samples include fluids, such as whole blood, serum or plasma, tissues, cell samples, tissue biopsies.

Preferred samples are ocular cell or ocular tissue samples, whole blood, serum or plasma.

The method of the invention may be used in a prenatal method for predicting ocular anterior chamber dysgenesis or a disorder resulting from ocular anterior chamber dysgenesis such as inherited congenital microcoria (MCOR) or glaucoma.

In an embodiment, the disorder resulting from ocular anterior chamber dysgenesis is inherited congenital microcoria (MCOR).

In an embodiment, the disorder resulting from ocular anterior chamber dysgenesis is developmental glaucoma.

By testing a subject according to the method of the invention, ocular anterior chamber dysgenesis may be diagnosed early and then the subject may be treated to slow or stop the evolution of disorders resulting therefrom.

The expression "defective *GPR180* gene" should be understood broadly. It encompasses a mutated *GPR180* gene which results in a non-functional G protein-coupled receptor 180, the absence of the *GPR180* gene and/or a distant element regulating the gene itself and/or surrounding genes comprised between hg19 position chromosome 13:95241606-95276905. A "defective *GPR180* gene" may also result from an abnormally low expression of the *GPR180* gene in a tissue.

A *GPR180* mutation according to the invention may be found in a regulating region of *GPR180* gene (e.g. a promoter sequence, or a binding site for transcription factor), in introns or in exons that encode the GPR180 protein.

In an embodiment, the *GPR180* mutation is a mutation which results in a truncated GPR180 protein, a GPR180 mislocalization or in a reduction of *GPR180* expression or in the modification of the expression of surrounding genes.

GPR180 mutations may be detected by analyzing a *GPR180* nucleic acid molecule. In the context of the invention, *GPR180* nucleic acid molecules include mRNA, genomic DNA and cDNA derived from mRNA. DNA or RNA can be single stranded or double stranded.

DNA may be extracted using any methods known in the art, such as described in Sambrook et al., 1989.

RNA may also be isolated, for instance from tissue biopsy, using standard methods well known to the one skilled in the art such as guanidium thiocyanate-phenol-chloroform extraction.

GPR180 mutations may be detected in a RNA or DNA sample, preferably after amplification. For instance, the isolated RNA may be subjected to coupled reverse transcription and amplification, such as reverse transcription and amplification by polymerase chain reaction (RT-PCR), using specific oligonucleotide primers that are specific for a mutated site or that enable amplification of a region containing the mutated site. According to a first alternative, conditions for primer annealing may be chosen to ensure specific reverse transcription (where appropriate) and amplification; so that the appearance of an amplification product be a diagnostic of the presence of a particular *GPR180* mutation. Otherwise, RNA may be reverse-transcribed and amplified, or DNA may be amplified, after which a mutated site may be detected in the amplified sequence by hybridization with a suitable probe or by direct sequencing, or any other appropriate method known in the art. For instance, a cDNA obtained from RNA may be cloned and sequenced to identify a mutation in *GPR180* sequence.

Actually numerous strategies for genotype analysis are available (Antonarakis et al., 1989; Cooper et al., 1991; Grompe, 1993). Briefly, the nucleic acid molecule may be tested for the presence or absence of a restriction site. When a base substitution mutation creates or abolishes the recognition site of a restriction enzyme, this allows a simple direct PCR test for the mutation. Further strategies include, but are not limited to, direct sequencing, restriction fragment length polymorphism (RFLP) analysis; hybridization with allele-specific oligonucleotides (ASO) that are short synthetic probes which hybridize only to a perfectly matched sequence under suitably stringent hybridization conditions; allele-specific PCR; PCR using mutagenic primers; ligase-PCR, HOT cleavage; denaturing gradient gel electrophoresis (DGGE), temperature denaturing gradient gel electrophoresis (TGGE), single-stranded conformational polymorphism (SSCP) and denaturing high performance liquid chromatography (Kuklin et al., 1997). Direct sequencing may be accomplished by any method, including without limitation chemical sequencing, using the Maxam-Gilbert method ; by enzymatic sequencing, using the Sanger method ; mass spectrometry sequencing ; sequencing using a chip-based technology; and real-time quantitative PCR. Preferably, DNA from a subject is first subjected to amplification by polymerase chain reaction (PCR) using specific amplification primers. However

several other methods are available, allowing DNA to be studied independently of PCR, such as the rolling circle amplification (RCA), the Invader™ assay, or oligonucleotide ligation assay (OLA). OLA may be used for revealing base substitution mutations. According to this method, two oligonucleotides are constructed that hybridize to adjacent sequences in the target nucleic acid, with the join sited at the position of the mutation. DNA ligase will covalently join the two oligonucleotides only if they are perfectly hybridized.

Therefore, useful nucleic acid molecules, in particular oligonucleotide probes or primers, according to the present invention include those which specifically hybridize the regions where the mutations are located.

Oligonucleotide probes or primers may contain at least 10, 15, 20 or 30 nucleotides. Their length may be shorter than 400, 300, 200 or 100 nucleotides.

According to a further embodiment said mutation in the *GPR180 gene* may be detected at the protein level.

Said mutation may be detected according to any appropriate method known in the art. In particular a biological sample obtained from a subject may be contacted with antibodies specific of the mutated form of GPR180, i.e. antibodies that are capable of distinguishing between a mutated form of GPR180 and the wild-type protein (or any other protein), to determine the presence or absence of a GPR180 specified by the antibody.

Antibodies that specifically recognize a mutated GPR180 also make part of the invention. The antibodies are specific of mutated GPR180, that is to say they do not cross-react with the wild-type GPR180.

The antibodies of the present invention may be monoclonal or polyclonal antibodies, single chain or double chain, chimeric antibodies, humanized antibodies, or portions of an immunoglobulin molecule, including those portions known in the art as antigen binding fragments Fab, Fab', F(ab')₂ and F(v). They can also be immunoconjugated, e.g. with a toxin, or labelled antibodies.

Whereas polyclonal antibodies may be used, monoclonal antibodies are preferred for they are more reproducible in the long run.

Procedures for raising "polyclonal antibodies" are also well known. Polyclonal antibodies can be obtained from serum of an animal immunized against the appropriate antigen, which may be produced by genetic engineering for example according to standard methods well-known by one skilled in the art. Typically, such antibodies can be raised by administering mutated GPR180 subcutaneously to New Zealand white rabbits which have first been bled to obtain pre-immune serum. The antigens can be injected at a total volume of 100 µl per site at six different sites. Each injected material may contain adjuvants with or without pulverized acrylamide gel containing the protein or polypeptide after SDS-polyacrylamide gel electrophoresis. The rabbits are then bled two weeks after the first injection and periodically boosted with the same antigen three times every six weeks. A sample of serum is then collected 10 days after each boost. Polyclonal antibodies are then recovered from the serum by affinity chromatography using the corresponding antigen to capture the antibody.

This and other procedures for raising polyclonal antibodies are disclosed by Harlow et al. (1988) which is hereby incorporated in the references.

A "monoclonal antibody" in its various grammatical forms refers to a population of antibody molecules that contains only one species of antibody combining site capable of immunoreacting with a particular epitope. A monoclonal antibody thus typically displays a single binding affinity for any epitope with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different epitope, e.g. a bispecific monoclonal antibody. Although historically a monoclonal antibody was produced by immortalization of a clonally pure immunoglobulin secreting cell line, a monoclonally pure population of antibody molecules can also be prepared by the methods of the present invention.

Laboratory methods for preparing monoclonal antibodies are well known in the art (see, for example, Harlow et al., 1988). Monoclonal antibodies (mAbs) may be prepared by immunizing purified mutated GPR180 into a mammal, e.g. a mouse, rat, human and the like mammals. The antibody-producing cells in the immunized mammal are isolated and fused with myeloma or heteromyeloma cells to produce hybrid cells (hybridoma). The hybridoma cells producing the monoclonal antibodies are utilized as a source of the desired monoclonal antibody. This standard method of hybridoma culture is described in Kohler and Milstein (1975).

While mAbs can be produced by hybridoma culture the invention is not to be so limited. Also contemplated is the use of mAbs produced by an expressing nucleic acid cloned from a hybridoma of this invention. That is, the nucleic acid expressing the molecules secreted by a hybridoma of this invention can be transferred into another cell line to produce a transformant. The transformant is genotypically distinct from the original hybridoma but is also capable of producing antibody molecules of this invention, including immunologically active fragments of whole antibody molecules, corresponding to those secreted by the hybridoma. See, for example, U.S. Pat. No. 4,642,334 to Reading; PCT Publication No.; European Patent Publications No. 0239400 to Winter et al. and No. 0125023 to Cabilly et al.

Antibody generation techniques not involving immunisation are also contemplated such as for example using phage display technology to examine naive libraries (from non-immunised animals); see Barbas et al. (1992), and Waterhouse et al. (1993).

Antibodies raised against mutated GPR180 may be cross reactive with wild-type GPR180. Accordingly a selection of antibodies specific for mutated GPR180 is required. This may be achieved by depleting the pool of antibodies from those that are reactive with the wild-type GPR180, for instance by submitting the raised antibodies to an affinity chromatography against wild-type GPR180.

Alternatively, binding agents other than antibodies may be used for the purpose of the invention. These may be for instance aptamers, which are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity. Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library,

each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of this class of molecules have been reviewed in Jayasena S.D., 1999. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as *E. coli* Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996).

Probe, primers, aptamers or antibodies of the invention may be labelled with a detectable molecule or substance, such as a fluorescent molecule, a radioactive molecule or any others labels known in the art. Labels are known in the art that generally provide (either directly or indirectly) a signal.

The term "labelled", with regard to the probe, primers, aptamers or antibodies of the invention, is intended to encompass direct labelling of the the probe, primers, aptamers or antibodies of the invention by coupling (i.e., physically linking) a detectable substance to the the probe, primers, aptamers or antibodies of the invention, as well as indirect labeling of the probe, primers, aptamers or antibodies of the invention by reactivity with another reagent that is directly labeled. Other examples of detectable substances include but are not limited to radioactive agents or a fluorophore (e.g. fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or Indocyanine (Cy5)). Examples of indirect labeling include detection of a primary antibody using a fluorescently labeled secondary antibody and end-labeling of a DNA probe with biotin such that it can be detected with fluorescently labeled streptavidin. An antibody or aptamer of the invention may be labelled with a radioactive molecule by any method known in the art.

Typically, the *GPR180* mutation may be detected by contacting the DNA of the subject with a nucleic acid probe, which is optionally labeled.

Primers may also be useful to amplify or sequence the portion of the *GPR180 gene* containing the mutated positions of interest.

Such probes or primers are nucleic acids that are capable of specifically hybridizing with a portion of the *GPR180 gene* sequence containing the mutated positions of interest. That means that they are sequences that hybridize with the portion mutated *GPR180* nucleic acid sequence to which they relate under conditions of high stringency.

Typically the deletion of the *GPR180 gene* may be detected by commonly used techniques such as comparative genomic hybridization (GCH).

The invention will further be illustrated in view of the following figures and example.

Legends to tables and figures

Table 1. Summary of 13q32.1 deletions identified by array CGH and characterized by sequencing of the intervening segments. †denotes a partial deletion of the gene. Sequences shared between the proximal and distal sequences at the junction are underlined. The breakpoint (/)

has been arbitrary placed at the 5' of the identical sequences. Nucleotide positions refer to the human genome reference sequence (hg19 assembly) available at UCSC (genome.ucsc.edu).

Figure 1. Pedigrees of the six families segregating MCOR. Autosomal dominant transmission is supported by father to son transmission in all six families. Individual numbers in pedigrees FR1⁹ and JP1⁶ are those previously reported. Available DNAs can be identified by the presentation of their genotype at the 13q32.1 locus (Del: deletion, +: wildtype allele). Individuals affected with MCOR who were examined by gonioscopy (asterisk) had all iridocorneal angle dysgenesis.

Figure 2. Overview of the 13q32.1 deletions identified in MCOR. Overview of the 13q32.1 locus (chr13:95,110,000-95,375,000; hg19) with custom tracks showing the delineated deletions presented in this study (horizontal grey bars). At the top, the RefSeq Genes Track is included. In addition, ENCODE and conservation tracks are displayed.

Figure 3. Pedigree, *GPR180* genotypes and gonioscopic aspects in family FR3 and FR1. The iris spicules (arrows) consistent with an abnormal development of the iridocorneal angle are seen in individuals harboring the *GPR180* c.343C>T (p.Gln115*) mutation only as well as in individual FR1_V1 affected with microcoria. MCOR: microcoria; GD: goniodysgenesis; LCA: Leber congenital amaurosis

EXAMPLE

Submicroscopic deletions at 13q32.1 cause congenital microcoria

Abstract

Congenital microcoria (MCOR) is a rare autosomal dominant disorder characterized by inability of the iris to dilate owing to absence of dilator pupillae muscle. So far, a dozen MCOR families are reported worldwide. By using whole-genome oligonucleotide array CGH, we have identified deletions at 13q32.1 segregating with MCOR in six families originating from France, Japan and Mexico. Breakpoint sequence analyses showed nonrecurrent deletions in 5/6 families. The deletions varied from 35 Kbp to 80 Kbp in size, but invariably encompassed or interrupted only two genes: TGDS encoding the TDP-glucose 4,6-dehydratase and GPR180 encoding the G protein-coupled receptor 180, also known as intimal thickness-related receptor (ITR). Unlike TGDS which has no known function in muscle cells, GPR180 is involved in the regulation of smooth muscle cell growth. The identification of a null GPR180 mutation segregating over two generations with iridocorneal angle dysgenesis which can be regarded as a MCOR endophenotype is consistent with the view that deletions of this gene are the cause of ocular anterior chamber dysgenesis.

Here, we report a study combining Sanger sequencing and array comparative genomic hybridization (CGH) which allowed the identification of the molecular defect underlying the disease at the 13q31-32 locus.

We obtained DNA samples of affected and unaffected members of six MCOR families originating from France, Japan and Mexico (FR1-2, JP1-2, MX1-2, respectively). Three out of these families were previously reported (FR1, the original family which allowed mapping of the MCOR locus on chromosome 13q31-q32,^{9,10} JP1⁶ and MX1⁴). The pedigrees of the families are presented in Figure 1. The study was approved by Ethics committees of each participating Institutions, namely *Paris Ile-de-France II*, University of Occupational and Environmental Health, Japan and Instituto de Oftalmologia Conde de Valenciana, Mexico City. Individuals participating to the study provided informed consents for molecular analyses.

Sanger sequencing of the coding region and intron-exon boundaries of genes lying within the 8 Mb MCOR interval on 13q31-q32, and whole exome sequencing combined with linkage analysis failed to detect candidate disease-causing variants segregating with the disease in families FR1 and JP1, respectively. Considering the strong linkage at the locus in FR1 ($Z_{\max} = 9.79$, $\Theta = 0$),⁹ we assumed that the mutation in this family was present in an unscreened region or that it might consist in a genomic rearrangement undetectable using PCR-based screening methods. To assess this latter hypothesis, we subjected the DNA of an affected individual (FR1_III7, Figure 1) to comparative genomic hybridization (CGH) on high resolution oligonucleotide microarray (Affymetrix Cytogenetics Whole-Genome 2.7M Array). Calculation of test over reference Log2 intensity Ratios identified a 54.8 Kbp deletion in the 13q32.1 region (Figure 2). We amplified the junction fragment by subjecting the genomic DNA of the index case to PCR using primers designed just outside of the predicted deletion boundaries. Direct sequencing of the 1.1 Kb intervening segment showed that the deletion extended from 95,227,374 to 95,277,864 (positions on chromosome 13 according to the Genome Reference Consortium Human Build 37) with centromeric and telomeric breakpoints in intron 11 and intron 8 of the tail-to-tail genes: TDP-glucose 4,6-dehydratase (*TGDS*, NM_014305.2) and the G protein-coupled receptor 180 (*GPR180*, [NM_180989.5], also known as intimal-thickness-related receptor, *ITR* [MIM607787]), respectively (Table 1 and Figure 2).

Interestingly, multiplex PCR of short fluorescent fragments (QMPSF)¹³ using primer pairs specific to *GPR180*, *TGDS* and a control gene (*CFTR*, NM_000492.3) suggested hemizyosity at 13q32.1 in the other French family as well as the two Japanese and two Mexican MCOR families. In all five families, array CGH confirmed the presence of 13q32.1 deletions, which estimated sizes ranged from 39 Kbp to 88.9 Kbp. Direct sequencing of intervening segments amplified using primers designed just outside of the predicted breakpoints showed that the deletions extended from 95,241,606 to 95,276,905 (35.3 Kbp encompassing 4/12 *TGDS* and 7/9 *GPR180* exons), 95,228,262 to 95,300,908 (72.6 Kbp encompassing 11/12 *TGDS* exons, *GPR180* and *mir_562*), 95,236,251 to 95,309,380 (73.1 Kbp encompassing 4/12 *TGDS* exons, *GPR180* *mir_562* and *5S_rRNA*) and 95,225,217 to 95,305,083 (79.9 Kbp encompassing *GPR180*, *mir_562* and *5S_rRNA*) in families FR2, JP1, JP2 and MX1, respectively (Table 1, Figure 2).

In family MX2, we failed to amplify the intervening segment using primers designed with array CGH data. Considering that MX1 and MX2 families had the same ethnic background and shared the same predicted distal deletion breakpoint (Table 1) we assumed that both families could have inherited the same deletion by descent and that, in corollary, lack of amplification of the junction fragment in family MX2 could result from incorrectly predicted proximal deletion breakpoint. Using the primers designed to amplify family MX1 junction fragment, we were able to amplify family MX2 intervening segment. Direct sequencing demonstrated the two families shared the exact same deletion (Table 1 and Figure 2). Analysis of microsatellite markers of chromosome 13q31-q32 showed that the deletion was carried by a common 6.4 Mb haplotype suggesting that it might have been transmitted within both families by a common ancestor.

Deletion fragment-specific PCR assays based on the amplification of intervening segments in all available DNA samples (see Figure 1) allowed us to confirm the co-segregation of the deletions with the disease in 4/6 families. Segregation analysis could not be performed in Families FR2 and JP2 because of a lack of DNA samples. Positive PCR amplification of intragenic *GPR180* fragments confirmed heterozygosity of all deletions (not shown). None of the deletions identified here have been reported in the Database of Genomic Variants (DGV) nor in the cohort of individuals affected with variable diseases analyzed by array CGH at our Institute (n = 96). However, chromosome 13q deletions are not uncommon and cause a wide spectrum of phenotypes correlated to the size and position of the deleted region. To our knowledge, microcoria has not been described in individuals with 13q deletions encompassing the 13q32.1 region. However, microcoria could be overlooked or could not manifest owing to severe eye dysgenesis since about one third of children with 13q deletion syndrome have iris and choroid coloboma, glaucoma, cataracts, and cloudy lenses. Together, these data are consistent with the causality of 13q32.1 deletions in MCOR.

Inspection of sequences surrounding MCOR deletion breakpoints identified a duplicated sequence prone to recurrent nonallelic homologous recombination (NAHR) in a unique family (FR2, 37bp sequence shared between *TGDS* intron 4 and *GPR180* intron 7 at positions 95,241,662-95,241,698 and 95,276,905-95,276,998, respectively).

Recently, several microhomology-mediated repair mechanisms have been described in the etiology of non-recurrent CNVs in human disease.¹⁴⁻¹⁶ These mechanisms, which are guided by the surrounding genomic architecture, include microhomology-mediated end-joining (MMEJ),¹⁷ fork stalling and template switching (FoSTeS),¹⁸ microhomology-mediated break-induced replication (MMBIR),¹⁹ serial replication slippage (SRS),²⁰ and break-induced SRS (BISRS).²¹ Extensive bioinformatic analysis of MCOR deletion breakpoints and surrounding genomic architecture allowed the identification of perfectly matching 1 or 2 base pairs shared between the proximal and distal sequence at the junctions, sequence motifs and/or repetitive elements which are likely to stimulate the formation of the 13q32.1 deletions by increasing susceptibility of DNA breakage or promote replication fork stalling. These findings are consistent with the view that microhomology-mediated mechanisms underlie non-recurrent MCOR deletions in families FR1, JP1, JP2, MX1 and MX2.

The minimal common deletion disrupted *GPR180* and *TGDS* (Table 1 and Figure 2), raising the possibility that haploinsufficiency of one or the two genes, or alternatively the loss of regulatory elements, might give rise to the phenotype.

GPR180 encodes a 201 amino-acid G protein-coupled receptor²² of the Rhodopsin-like receptors family which includes hormones, neurotransmitters, and light receptors, all of which transduce extracellular signals upon interaction with guanine nucleotide-binding proteins and activating ligands.²³ *GPR180* has been reported to be produced predominantly in vascular smooth muscle cells where its expression is upregulated in response to experimental injury.²⁴ The significant suppression of DNA synthesis and inability to produce neointima in response to vascular injury in the *Gpr180*^{-/-} mouse suggest that upregulation of *Gpr180* signaling contributes to vascular smooth muscle growth.²² In addition, gene expression profiling in normal human tissues have shown that it is highly expressed in myoepithelia (salivary gland, endomyometrium, prostate, lung and liver).²⁵ In the eye, *GPR180* is less abundant than in myoepithelia. However, it is listed in the top 20 genes having a significantly higher expression in the iris compared to the other ocular structures.²⁶ Hence, considering that the dilator pupillae arises during embryonic life by the differentiation of iris epithelial cells into myoepithelial cells,^{27,28} *GPR180* was regarded as a strong candidate MCOR gene.

The *Gpr180*^{-/-} mouse had resistance to experimental thickening of the intima but normal appearance, growth rate, reproduction, and histology of major organs.²² We examined *Gpr180*^{-/-} and *Gpr180*^{+/-} mice for anterior segment development and iris function. We found that both heterozygote and homozygote *Gpr180*-null eyes were undistinguishable from adult age-matched controls. In particular, we found no iris transillumination and normal drug-mediated mydriasis both in heterozygote and homozygote *Gpr180*-null mice. However, inspection of our in house exome database (>4200 exomes) for *GPR180* nonsense or frameshift variants with an acceptable amount of reads (≥ 10) identified a unique heterozygote *GPR180* nonsense mutation (c.343 C>T; p.Gln115*). This variant was found in our own series of individuals with neonatal retinal dystrophy, namely Leber congenital amaurosis (LCA, [MIM204000]). Interestingly, the ophthalmologic file of the blind individual harboring the p.Gln115* substitution (II2, family FR3; Figure 3) mentioned an abnormal iridocorneal angle at examination of the anterior segment of the eye. The individual, her parents and siblings consented to ophthalmological examination and genetic analysis. Iridocorneal angle dysgenesis was evidenced in all family members but the mother and a brother affected with LCA (family FR3; Figure 3). Evidence of father-to-son transmission demonstrated autosomal dominant transmission of the iridocorneal defect which segregated with the p.Gln115* substitution, independently from the autosomal recessive retinal disease (Figure 3). Whole-genome SNP genotyping data generated using Affymetrix GeneChip Human Mapping 10K 2.0 Arrays were available in this family. Retrospective analysis for linkage with the autosomal dominant anterior segment dysgenesis pointed 15 candidate chromosomal regions, including a region on 13q32.1 containing *GPR180*. Retrospective analysis of exome data found no heterozygote loss-of-function variants in any of these regions other than the *GRP180* p.Gln115* substitution, supporting the role of *GPR180* in the development of the irido-corneal angle. Nevertheless, none of the five individuals with both iridocorneal angle dysgenesis and the p.Gln115*

substitution had abnormal pupillary response or iris transillumination. Considering that iridocorneal angle dysgenesis is a constant symptom in congenital microcoria linked to 13q32.1 (31/31, 5/5, 1/1, 2/2 and 3/3 of examined MCOR individuals in families FR1^{5,9}, JP1⁶, JP2, MX1 and MX2, respectively; Figures 1 and 3), goniodysgenesis in family FR3 may be regarded as a MCOR endophenotype.

- 5 The reason why heterozygosity for the p.Gln115* substitution was not sufficient to cause the full range of MCOR symptoms could reside in the production of a truncated protein retaining some of its function. Alternatively, premature termination codon (PTC) self-correcting mechanisms could be involved, in particular translational read-through which has recently been reported to be more abundant than expected in higher species, including human.²⁹ Nonsense-associated altered splicing (NAS), which
- 10 consists in selective exclusion of an in-frame exon with a premature termination codon, is another possible correcting mechanism.³⁰⁻³³ Recently, a nonsense mutation in *CEP290* which mutations cause blindness has been shown to induce exon skipping and to lead to a relatively mild retinal phenotype.³⁴ Because the skipping of *GPR180* exon 2 would not disrupt the open reading frame, NAS could explain why the p.Gln115* substitution is not as detrimental as gene ablation.
- 15 The p.Gln115* substitution was not reported in the Exome Aggregator database (ExAC), Exome Variant Server (EVS), 1000Genome and dbSNP datasets. However, we identified in the ExAC database twelve other rare variants which may cause protein truncation (6 nonsense and 6 frameshift mutations, 0.00004 <minor allele frequency <0.000008; Table 2). Provided that these variants are confirmed, studying their molecular consequence at the mRNA and/or the protein levels and, knowing
- 20 the ophthalmologic status of carrier individuals will certainly help in understanding the role of *GPR180* in MCOR and goniodysgenesis. Meanwhile, to address this important question, we screened the *GPR180* exome for mutations in a series of individuals having an eye disease with goniodysgenesis, including ten individuals with Axenfeld-Rieger anomaly or Peters anomaly [MIM604229] and no *PAX6*, *PITX2*, *FOXC1*, *CYP1B1*, *MAF* or *MYOC* mutation (n = 5 and n = 5, respectively), and eleven index
- 25 cases of primary congenital glaucoma (n = 9) or juvenile glaucoma (n = 2) with no *CYP1B1* mutations. Primer sequences are given in Table 3. No *GPR180* candidate disease variants were identified in *GPR180* exon and intron-exon boundaries in any of the individuals.

- The second gene lost or disrupted in MCOR individuals, *TGDS*, encodes the dTDP-D-glucose 4,6-dehydratase, an evolutionarily conserved NAD-dependent sugar epimerase/dehydratase of the short chain dehydrogenase/reductase extended-type family (SDR2E1, EC:4.2.1.46). In human, SDR enzymes display a wide substrate spectrum, ranging from steroids, alcohols, sugars, and aromatic compounds to xenobiotics.³⁵ *TGDS* catalyses the dehydration of dTDP-alpha-D-glucose into dTDP-4-dehydro-6-deoxy-alpha-D-glucose. It contributes to deoxy-sugar metabolism,^{35,36} but its exact function is unknown. In bacteria, *TGDS* is essential to the biogenesis of cell envelope components and
- 30 antibiotics,³⁷ while in *C. elegans*, it is required for normal growth rates, larval development and survival, reproduction and coordinated locomotion.³⁸ In human eyes, *TGDS* expression is lower than that of *GPR180*, and unlike this latter, it has no preferential expression in the iris²⁶. More importantly, very recently, biallelic *TGDS* mutations have been reported to cause Catel-Manzke syndrome [MIM302380] which is characterized by Pierre Robin sequence (MIM 261800; HP:0000201) and a unique form of
 - 35

bilateral hyperphalangy causing clinodactyly of the index finger (HP:0009467).³⁹ Neither the affected individuals, nor their heterozygote parents are reported to have eye disease.³⁹ Therefore, it is unlikely that TGDS dysfunction in human is responsible for MCOR.

In summary, here we report heterozygote 13q32.1 deletions in 6/6 MCOR families of variable ethnic origin, which invariably encompass *GPR180* and *TGDS*. We demonstrate that *GPR180* ablation is the cause of the disease. It provides insights into the development of the anterior chamber of the eye, which anomalies are an important cause of visual loss due to glaucoma.

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		Deletions predicted by Array CGH					Actual deletions			
Families	Individuals	Centromeric boundaries					Telomeric boundaries			
		+Probe (position)	-Probe (position)	-Probe (position)	+Probe (position)	Estimated size (Kbp)	Deletion breakpoints	Size (Kbp)	Genes included or disrupted by the deletion	Sequence of the junction fragment
FR1	III7	C-08UO9 95,224,547	C-08UOA 95,225,723	S-2NJCM 95,276,735	C-08UPK 95,279,378	54.8	95,227,374-95,277,864	50.5	<i>TGDS</i> †, <i>GPR180</i> †	AAAAATCAACTATTTTCTTCTTAACTTC TAAAGTCATTCATTAAGTGAACCTTGGT/ <u>ATGC</u> AAATATGAATGTACATTTCTTTTCTTTTAC AGGAATATTACACATTGTG (SEQ ID NO: 1)
FR2	II1	C-3ZRLF 95,238,814	C-3DRMS 95,238,827	C-6YHLK 95,277,799	C-4AEYA 95,277,841	39.0	95,241,606-95,276,905	35.2	<i>TGDS</i> †, <i>GPR180</i> †	TCACCAGGCTGGAGTGCAGTGGTCAACCT CAGCTCACTGCAAGCTCTGTCTCC/ <u>GGGTT</u> <u>CACGCCATTCTCTGCCCTCAGCCTCCCGAGT</u> <u>AACTCGGGCCACGCCGGGCTAAGTTTGT</u> ATTTTAGTAGAGAGCGGGGTTTCCCG (SEQ ID NO: 2)
JP1	IV1	S-4RUHL 95,211,097	C-7G8VT 95,211,706	C-5TEUV 95,299,777	C-5TIVX 95,300,029	88.9	95,228,262-95,300,908	72.6	<i>TGDS</i> †, <i>GPR180</i> , <i>mir_562</i>	ATCAATTTTACATACATGTAAAAAGAAA AAAGCTACAATAATAATTAAGACACCCAGT /GTCCTCTCTCAGCACAGCAGTTTACTTCTT CAGGGCAGCAGGAGAACTCTCTCTGACTTC (SEQ ID NO: 3)
JP2	III7	C-3ERLF 95,238,800	C-3DRMJ 95,238,827	S-4OHPW 95,309,706	C-6MYDV 95,309,751	70.9	95,236,251-95,309,380	73.1	<i>TGDS</i> †, <i>GPR180</i> , <i>mir_562</i> , <i>5S_rRNA</i>	CGATGATCAATGTCTACTTACAGTAAGAAAA CCCAAAATTAAGAACTCAGAGATAC/TCTCAT TCTCCAGCTGAAATCTCAGAAAATAATGTCT ATGCCATGTAATTTTCCCC (SEQ ID NO: 4)
MX1	II3	S-3HRGB 95,218,014	C-6DQEK 95,219,578	C-6IBIF 95,305,254	C-4DISK 95,305,294	87.2	95,225,217-95,305,083	79.9	<i>TGDS</i> , <i>GPR180</i> , <i>mir_562</i> , <i>5S_rRNA</i>	AACCAACTGAAAGGAGAAAAAGTTGATCT TAGTTTATAGATGGATTGGCTGTTC/ <u>TGAG</u> CCCCATAACAGTGAGCACCTCTAGCACCTAG GATGCCAGCTGCATGTAT (SEQ ID NO: 5)
MX2	III1	C-7ALKO 95,227,834	C-3GDL5 95,227,835	C-6IBIF 95,305,254	C-4DISK 95,305,294	77.5	95,225,217-95,305,083	79.9	<i>TGDS</i> , <i>GPR180</i> , <i>mir_562</i> , <i>5S_rRNA</i>	AACCAACTGAAAGGAGAAAAAGTTGATCT TAGTTTATAGATGGATTGGCTGTTC/ <u>TGAG</u> CCCCATAACAGTGAGCACCTCTAGCACCTAG GATGCCAGCTGCATGTAT (SEQ ID NO: 6)

Table 1.

Chromosome:position_ variant	Protein consequence	Allele Count	Allele Number	Number of Homozygotes	Allele Frequency
13:95254225_G/A	p.Trp15Ter	1	11290	0	0.00008857
13:95254299_C/T	p.Gln40Ter	1	17616	0	0.00005677
13:95271542_T/A	p.Leu215Ter	2	122854	0	0.00001628
13:95271735_G/T	p.Gly234Ter	1	122540	0	0.000008161
13:95278239_T/A	p.Leu369Ter	1	122674	0	0.000008152
13:95278286_C/T	p.Gln385Ter	3	122444	0	0.00002450
<u>13:95271434_T/TA</u>	p.Val180SerfsTer18	1	122770	0	0.000008145
<u>13:95273380_T/TATGC</u>	p.Gly265HisfsTer45	1	122930	0	0.000008135
<u>13:95273411_GA/G</u>	p.Lys273SerfsTer24	5	122932	1	0.00004067
<u>13:95275395_T/TA</u>	p.Ile310AsnfsTer5	3	122712	0	0.00002445
<u>13:95275491_C/CT</u>	p.Tyr342LeufsTer41	1	122908	0	0.000008136
<u>13:95279324_CTCTT/C</u>	p.Phe409CysfsTer15	1	122876	0	0.000008138

Table 2: Rare *GPR180* variants predicted to truncate the protein. Inspection of ExAC, EVS, 1000Genome and dbSNP datasets pointed 12 nonsense and frameshift variants in the ExAC database, including one homozygote change, which reality needs to be demonstrated by Sanger sequencing.

5

Exon	Forward primer (5'-3')(SEQ ID No.)	Reverse primer (5'-3') (SEQ ID No.)
1	ggcctctgactggggaatgt (7)	aggtgaggctggggaagt (8)
2	ttgcttctgaatgtgtgtca (9)	tgggattatgctgtcaggaa (10)
3	cacaaggctctggatttctgag (11)	ggaaacacagggtgctttca (12)
4	ccccaagggtacaaaattgtttaag (13)	gcaagcataatttgggtgact (14)
5	tcaaaaccactaaaactgtgttca (15)	tctgttctcagtcaagctgat (16)
6	tttgaaaatggcttttgtgg (17)	tgacacaaattgtagaagctcaga (18)
7	aaaattcatttgtctagcttaggg (19)	tcccaaagaaaccagaacca (20)
8	ggcctgttgaggtaatacaga (21)	aaactcaaattatctgcttattgg (22)
9	tccttaacaattagagtctgagca (23)	atgctctgctttcgctgttc (24)

Table 3. Primer pairs used to screen the *GPR180* gene for mutations in individuals with goniodysgenesis.

10

CLAIMS

1. A method for diagnosing ocular anterior chamber dysgenesis or predicting the risk of developing a disorder resulting from ocular anterior chamber dysgenesis, in a subject, said method comprising
5 detecting a defective *GPR180* gene encoding the G protein-coupled receptor 180 in a sample obtained from said subject, wherein the presence of a defective *GPR180* gene is indicative of ocular anterior chamber dysgenesis or of a risk of developing ocular anterior chamber dysgenesis.
2. The method of claim 1, wherein the method is a prenatal method.
- 10 3. A method for predicting a risk of a subject to transmit a disorder resulting from ocular anterior chamber dysgenesis to offspring, said method comprising detecting a defective *GPR180* gene in a sample obtained from said subject, wherein the presence of a defective *GPR180* gene is indicative of a risk of transmitting a disorder resulting from ocular anterior chamber dysgenesis.
- 15 4. A method according to any one of claims 1 to 3, wherein, the disorder resulting from ocular anterior chamber dysgenesis is inherited congenital microcoria (MCOR) or glaucoma.
5. The method according to claim 4, wherein the disorder resulting from ocular anterior chamber
20 dysgenesis is MCOR.
6. The method according to claim 4, wherein the disorder resulting from ocular anterior chamber dysgenesis is developmental glaucoma.

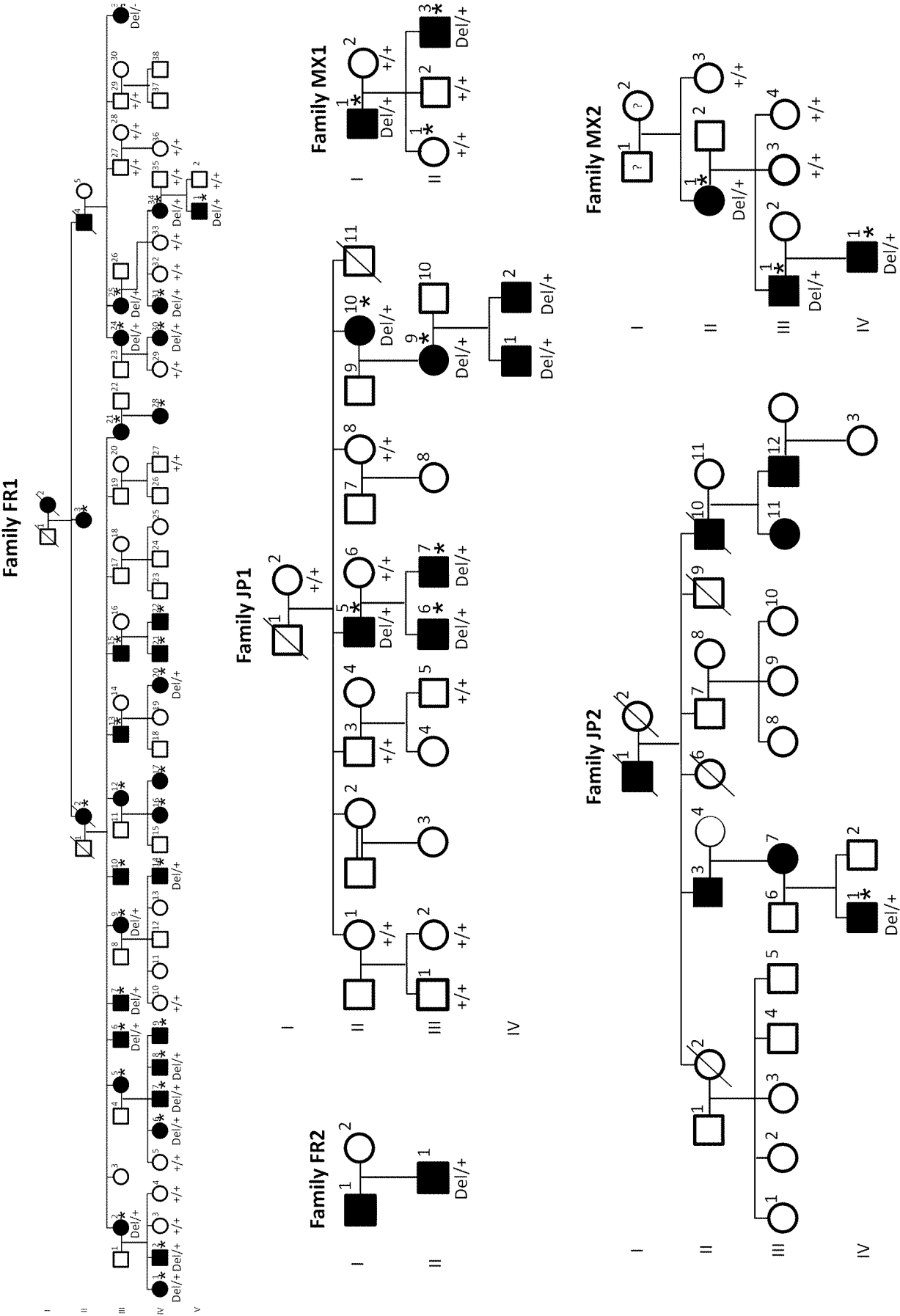


Figure 1

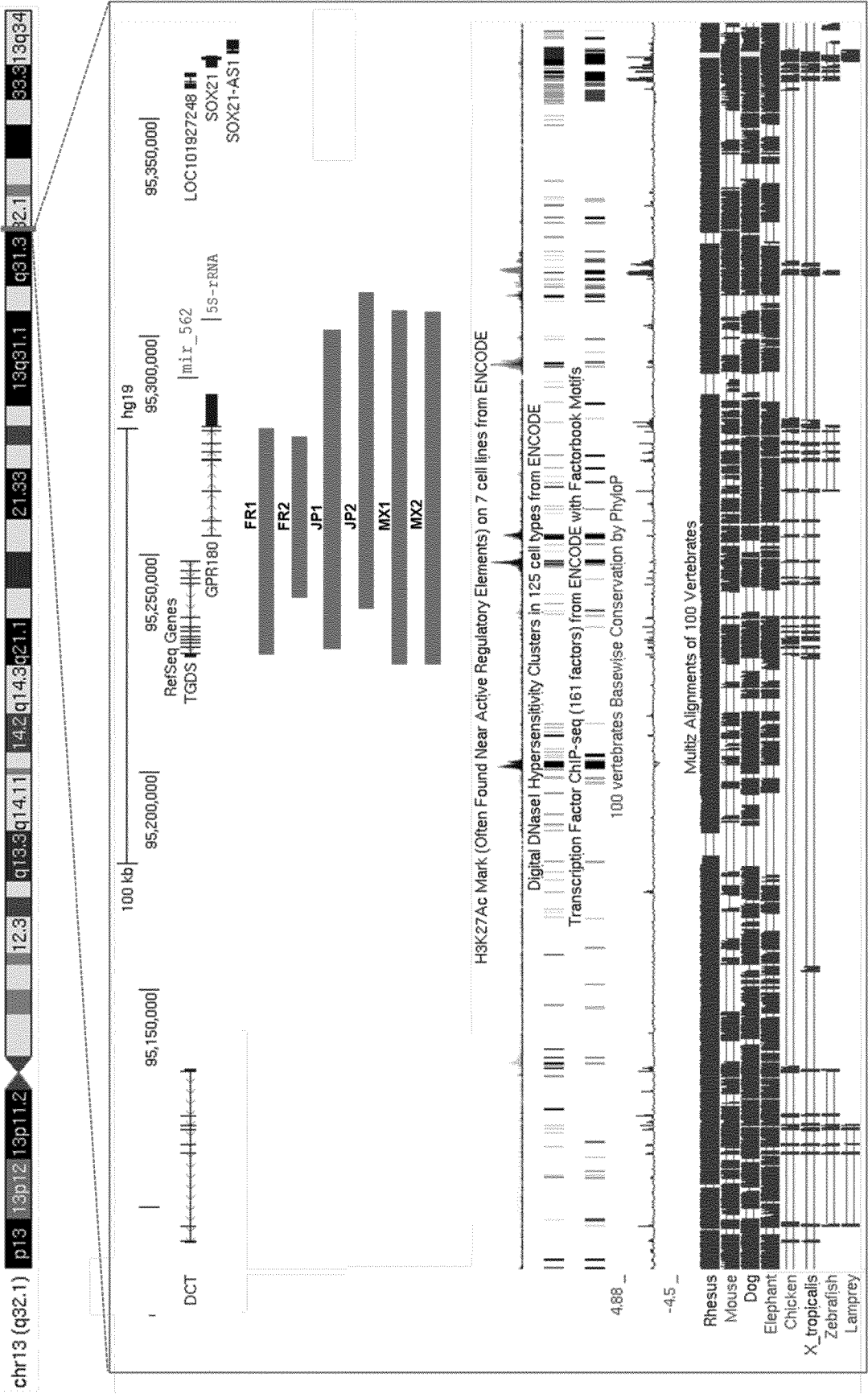


Figure 2

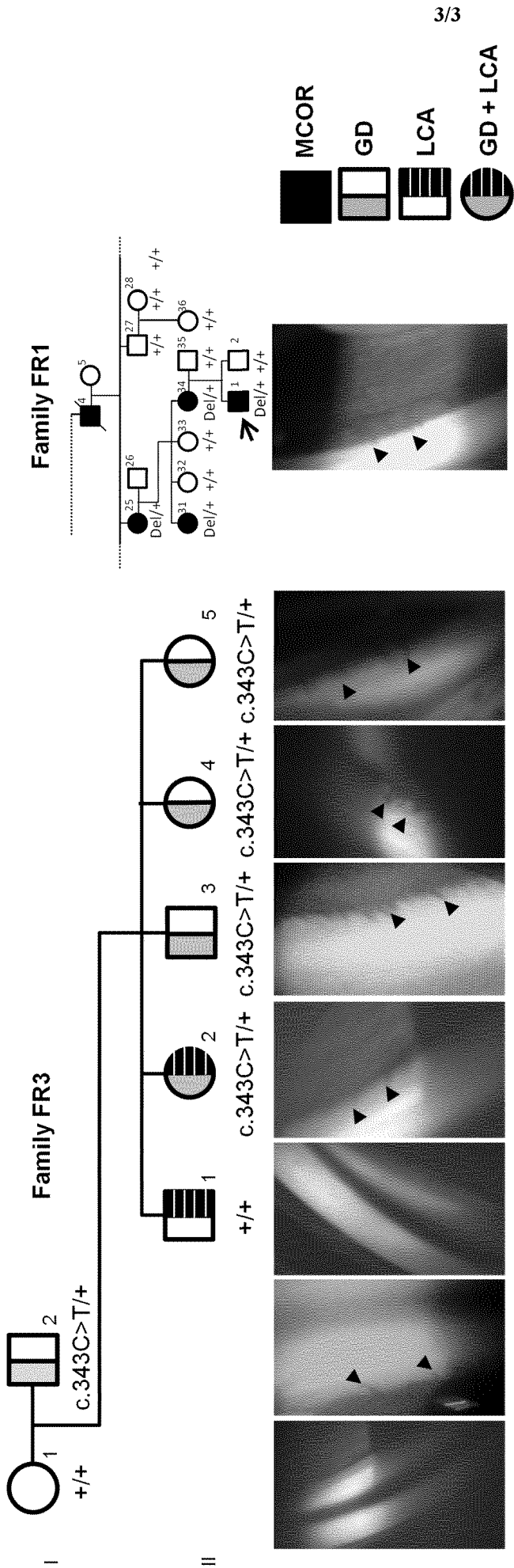


Figure 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/054927

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	RAMPRASAD VL; SRIPRIYA S; RONNIE G; NANCARROW D; SAXENA S; HEMAMALINI A; KUMAR D; VIJAYA L; KUMARAMANICKAVEL G: "Genetic homogeneity for inherited congenital microcoria loci in an Asian Indian pedigree", MOL VIS., vol. 11, 2005, pages 934-40, XP002744785, cited in the application the whole document ----- -/--	1-6



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

1 June 2016

Date of mailing of the international search report

09/06/2016

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Costa Roldán, Nuria

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/054927

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	V. NESIN ET AL: "Activating mutations in STIM1 and ORAI1 cause overlapping syndromes of tubular myopathy and congenital miosis", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 111, no. 11, 18 March 2014 (2014-03-18), pages 4197-4202, XP055214414, ISSN: 0027-8424, DOI: 10.1073/pnas.1312520111 abstract	1-6
A	----- J. WENG ET AL: "Deletion of G protein-coupled receptor 48 leads to ocular anterior segment dysgenesis (ASD) through down-regulation of Pitx2", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 105, no. 16, 22 April 2008 (2008-04-22), pages 6081-6086, XP055214360, ISSN: 0027-8424, DOI: 10.1073/pnas.0708257105 abstract	1-6
A	----- GILLESPIE R L ET AL: "The use of autozygosity mapping and next-generation sequencing in understanding anterior segment defects caused by an abnormal development of the lens", HUMAN HEREDITY JULY 2014 S. KARGER AG CHE, vol. 77, no. 1-4, July 2014 (2014-07), pages 118-137, XP002744786, ISSN: 0001-5652 abstract; table 4 page 128 - page 130	1-6
A	----- ITO YOKO A ET AL: "Genomics and anterior segment dysgenesis: a review.", CLINICAL & EXPERIMENTAL OPHTHALMOLOGY 2014 JAN-FEB, vol. 42, no. 1, January 2014 (2014-01), pages 13-24, XP002744787, ISSN: 1442-9071 page 13 - page 15 ----- -/--	1-6

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2016/054927

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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T	----- LUCAS FARES-TAIE ET AL: "Submicroscopic Deletions at 13q32.1 Cause Congenital Microcoria", THE AMERICAN JOURNAL OF HUMAN GENETICS, vol. 96, no. 4, 1 April 2015 (2015-04-01), pages 631-639, XP055214363, ISSN: 0002-9297, DOI: 10.1016/j.ajhg.2015.01.014 the whole document -----	1-6

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Information on patent family members

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