UNIVERSIDAD NACIONAL AUTÓNOMA DE MEXICO

Programa de Maestría y Doctorado en Ciencias Médicas, Odontológicas y de la Salud

Instituto de Oftalmología Conde de Valenciana

"IDENTIFICACIÓN DE VARIANTES GENÉTICAS CAUSANTES DE OFTALMOPATÍAS HEREDITARIAS UTILIZANDO ANÁLISIS GENÓMICOS"

TESIS DOCTORAL

QUE PARA OPTAR POR EL GRADO DE DOCTOR EN CIENCIAS MÉDICAS

Presenta

OSCAR FRANCISCO CHACÓN CAMACHO

Tutor

JUAN CARLOS ZENTENO RUÍZ FACULTAD DE MEDICINA

Miembros del comité tutoral

DRA. ROSENDA PEÑALOZA ESPINOSA

Universidad Autónoma Metropolitana, Unidad Xochimilco

DRA. ALESSANDRA CARNEVALE CANTONI

Instituto de Medicina Genómica

Ciudad de México, Noviembre de 2017

ÍNDICE

RESUMEN

Las enfermedades genéticas con un patrón de herencia monogénica son entidades raras pero que colectivamente representa un grupo importante de patologías que podrían ser responsables de hasta 10% de la morbi-mortalidad de la población general. Actualmente de conocen aproximadamente unas 6,000 enfermedades mendelianas de las cuales aproximadamente la mitad no tienen una base molecular identificada. Del total de estas enfermedades, más de un tercio incluyen afección ocular.

En la última década, el campo de la genética y genómica ha experimentado una revolución con la incorporación de tecnologías genómicas como lo es la utilización de los polimorfismos de un sólo nucleótido en las plataformas de microarreglos y el uso de la secuenciación de siguiente generación, herramientas centrales para el diagnóstico moderno de enfermedades mendelianas raras, con fenotipos no clásicos o con heterogeneidad genética. De este modo han colaborado al diagnóstico más rápido, más económico y preciso de cuadros clínicos que anteriormente representaban odiseas diagnósticas.

En este trabajo se presentan los resultados de estudios genómicos en tres oftalmopatías con patrón de herencia monogénica pero sin diagnóstico genético-molecular, incluyendo dos casos sin un diagnóstico clínico definido. Los resultados más importantes de estos estudios fueron la identificación de un nuevo gen en una de etas enfermedades y la obtención de diagnósticos clínicos y moleculares en otras dos enfermedades que habían sufrido odiseas diagnósticas. La utilización exitosa en estos casos de abordajes genómicos como el mapeo de homocigosidad de genoma completo y la secuenciación de exoma completo , demuestra la eficacia de estas metodologías para el estudio de oftalmopatías raras o con heterogeneidad genética .

INTRODUCCIÓN

Pocas áreas dentro de la Medicina Humana están teniendo grandes avances como la genética y la genómica. En el inicio de este siglo, la conclusión del Proyecto del Genoma Humana ha permitido conocer la secuencia completa del genoma humano (International Human Genome Sequencing Consortium, 2004), lo que a su vez ha contribuido enormemente a catalogar todos los genes humanos, a entender su estructura y su regulación, a determinar la variación de esos genes en distintas poblaciones, y por supuesto, a reconocer cómo la variación genética contribuye a las enfermedades humanas. Actualmente, el genoma de cualquier individuo puede ser caracterizado completamente, en lugar de estudiar un gen a la vez, y este logro está haciendo posible el crecimiento del campo de la medicina genómica. Al mismo tiempo, se han desarrollado diversas tecnologías genómicas, tales la como genotipificación con microarreglos y la secuenciación masiva de siguiente generación (NGS), que han permitido la identificación de genes causantes de enfermedad para entidades clínicas poco comunes o raras o con gran heterogeneidad genética.

HERENCIA MENDELIANA: ENFERMEDADES RARAS Y OFTALMOPATÍAS MONOGÉNICAS

En la práctica clínica, el principal objetivo de la genética es identificar el significado de las variaciones y mutaciones genéticas que predisponen a enfermedad, para modificar su evolución o impedir, incluso su aparición. Casi todas las enfermedades son el resultado de una acción combinada de los genes y el ambiente, pero el papel desempeñado por cada uno de estos factores puede ser mayor o menor. De este modo, hay tres tipos de enfermedades de acuerdo a su modo de herencia: cromosómicas, multifactoriales y monogénicas (Naussbaum y cols. 2015). En los trastornos cromosómicos el defecto no se debe a un error simple en la secuencia genética, sino a un exceso o un defecto de los genes contenidos en cromosomas completos o en fragmentos de éstos (Naussbaum y cols. 2015). Por su parte, los trastornos multifactoriales son responsables de la mayor parte de las enfermedades, y poseen susceptibilidad poligénica (muchos genes que tienen un pequeño efecto a menudo aditivo) e influencia del medio ambiente (King et al. 2002).

Las enfermedades monogénicas o Mendelianas están causadas por genes mutantes individuales. A pesar de que por separado son raras, como grupo las enfermedades mendelianas son responsables de una importante proporción de enfermedades y muertes. La prevalencia de los trastornos monogénicos en la población general es de un 2%, mientras que representa el 0.36% en recién nacidos. En la población de niños hospitalizados, probablemente el 6-8% presenta trastornos monogénicos (Baired y cols. 1998). En conjunto, los trastornos mendelianos se observan principalmente en la edad pediátrica, menos del 10% se manifiestan después de la pubertad y únicamente un 1% en la edad adulta (Naussbaum y cols. 2015).

Una alteración en un gen puede ser dominante o recesiva. Una mutación dominante sólo necesita estar presente en un miembro del par de alelos para tener un impacto clínico evidente. Por otro lado, una mutación de un gen recesivo se manifestará clínicamente cuando ambas copias de ese gen estén alteradas. Si la localización cromosómica del gen con la variante es dentro de un autosoma se denomina gen autosómico (o enfermedad autosómica), mientras que si está localizada en un gen situado en el cromosoma X, se dice que es ligado al X (Cassidy y cols. 2010). Datos actuales enlistan unas 6,115 enfermedades genéticas mendelianas, de las cuales 3,846 tienen una causa monogénica bien definida (https://www.omim.org/statistics/geneMap).

El término enfermedad rara es utilizado para describir condiciones que se presentan con una frecuencia igual o menor a 1 de cada 2,000 recién nacidos Aunque individualmente son raras, hay cerca de 5900 enfermedades genéticas que afectan a más del 10% de la población, representando así un impacto significativo para la salud. Muchas de estas enfermedades son hereditarias y son clasificadas como letales, severas no letales y no severas. Con las nuevas tecnologías de análisis genómicos, el diagnóstico de estas enfermedades es más rápido, menos caro y puede contribuir a la determinación del pronóstico así como también al tratamiento para la enfermedad (Pogue y cols. 2017).

ENFERMEDADES OCULARES MONOGÉNICAS

El ojo tiene un papel esencial en la evolución del estudio del genoma humano. Al menos 90% de los genes en el genoma humano son expresados en uno o más de los muchos tejidos del ojo y tipos celulares que lo conforman, en algún punto durante la vida de una persona. Congruente con esta impresionante “huella genómica” es la estadística acerca de que aproximadamente un tercio de las entradas en la base de datos Online Mendelian

Inheritance in Man (OMIM) refiere alguna estructura del ojo en la sinopsis clínica, lo que significa que la tercera parte de las enfermedades genéticas humanas tienen una repercusión en mayor o menor grado en el ojo (Sheffield y Stone, 2011). En las últimas 3 décadas se han mapeado y caracterizado más de 300 genes para enfermedades del ojo, con miles de mutaciones identificadas que causan directamente tales enfermedades. La estrategia de mapeo genético clásico, incluyendo investigaciones de genes candidatos y el análisis de ligamiento familiar a través de marcadores microsatélites o de SNPs distribuidos en todo el genoma, ha sido notablemente efectiva y exitosa para un amplio espectro de enfermedades del ojo, como en la retinosis pigmentaria (Nathans y Hogness, 1984), las distrofias corneales (Munier y cols. 1997), el retinoblastoma (Friend y cols. 1987), la neuropatía óptica de Leber (Singh y cols. 1989), y la amaurosis congénita de Leber (Marlhens y cols. 1997), entre otras.

ENFERMEDADES GENÉTICAS DE LA ÓRBITA: TELECANTO Y COLOBOMA DE PÁRPADO

Las malformaciones del ojo, la órbita y de los anexos oculares pueden ocurrir de manera aislada, en combinación con otras malformaciones oculares o craneofaciales, o como parte de síndromes con numerosas malformaciones sistémicas. Muchas de estas malformaciones pueden dañar severamente la visión, mientras otras sólo tienen significancia cosmética y otros no causan sintomatología y pueden ser diagnosticadas incidentalmente en una exploración rutinaria del ojo (Levin, 2003). Las anomalías congénitas pueden tener varias causas y aunque en un porcentaje notable de casos se deben a defectos en genes críticos del desarrollo normal, también pueden ser causadas por

alteraciones cromosómicas, teratógenos o complicaciones obstétricas como el oligohidramnios (Dollfus y Verloes, 2004).

Malformaciones que alteran la distancia interocular e interorbitaria Es importante distinguir entre los valores de normalidad y anormalidad para reconocer clínicamente diversas anomalías de la órbita y de los tejidos perioculares. Muchos defectos congénitos ocasionan una distancia anormal entre los ojos como característica cardinal. La distancia interorbitaria, que es la distancia más corta entre las paredes de la órbita, debe ser medida por medio de estudios de radiología o imagen para que la medición sea más precisa (Guercio y Martyn, 2007). En cada paciente deben ser medidas la distancia interpupilar, las distancias intercantal interna e intercantal externa, la longitud palpebral, y los resultados deben compararse con los valores normales (Dollfus y Verloes, 2004).

El telecanto es una anomalía definida por un aumento de la distancia entre los cantos internos, con distancia interpupilar normal y acompañada generalmente de aumento del tejido blando en la región frontonasal (Dollfus y Verloes, 2004). Varios síndromes genéticos incluyen telecanto, entre otros el síndrome de Axenfeld Rieger (principalmente el tipo 2) (Tumer y Bach-Holm, 2009), el síndrome de Barber-Say (Marchegiani y cols., 2015), el síndrome de Kaufman/Oculocerebrofacial (Kaufman y cols., 1971), el síndrome craneofacial-hipoacusia-mano (Gad y cols., 2008), el síndrome de microcórnea, miopía, telecanto y orejas rotadas (Khan, 2012), y el síndrome de Donnai-Barrow (Patel y cols., 2007), entre otros.

Por su parte, el coloboma de párpados se caracteriza por una fisura o grieta en el margen del párpado superior o inferior. Esta anomalía es usualmente triangular con la base en el margen del párpado y es de tamaño variable. Los colobomas situados en el párpado inferior pueden provocar ulceración de la córnea (Levin, 2003). El coloboma puede ser aislado o parte de síndrome congénitos y también ha sido descrito asociado a síndromes de bandas amnióticas (Miller y cols., 1987). Entre las entidades genéticas sindrómaticas con coloboma de parpados se encuentran el síndrome de Fraser (Hoefele y cols., 2013), el síndrome de Goldenhar/espectro oculoauriculovertebral (Strömland y cols., 2007), el síndrome de MOTA (Chacon-Camacho et al., 2017), el síndrome de ablefaron-macrostomía (De Maria y cols., 2016), el síndrome oculoectodérmico (Boppudi y cols., 2016), el síndrome de lipomatosis encéfalo-croneo-cutánea (Bennett y cols., 2016), y el síndrome de Schimmelpenning-Feurstein-Mims (Groesser y cols., 2012), entre otros.

CATARATA CONGÉNITA

La catarata es definida como una opacidad del cristalino que origina una variación de su índice refractivo. Esta variación resulta de cambios en la estructura de la lente lo que ocasiona una dispersión de la luz por la alta concentración de agregados proteicos (Messina-Baas y Cuevas-Covarrubias, 2017). Según la Organización Mundial de la Salud, en el mundo hay 253 millones de personas con discapacidad visual y 36 millones de personas con ceguera de los cuales la mayoría se encuentra en los países en desarrollo. Aproximadamente un cuarto de los casos de esta discapacidad visual y un tercio de los casos de ceguera se deben a cataratas no operadas (OMS: http://www.who.int/mediacentre/factsheets/fs282/es/). La catarata congénita tiene una prevalencia de 1-6 en 10,000 nacidos vivos y es una de las causas prevenibles de ceguera

en el mundo. En un 70% se presenta de forma aislada, mientras que 15% se asocia a otros defectos oculares, y 15% se acompaña de defectos sistémicos. Cerca de un 25% de las cataratas congénitas son hereditarias, particularmente cuando son bilaterales (Pichi y cols., 2016). La catarata congénita de tipo hereditario es genéticamente heterogénea y pueden presentar cualquier forma de patrón hereditario con más de 100 genes causantes de la enfermedad (Gillespie y cols., 2014). Así, las cataratas congénitas hereditarias pueden ser autosómicas dominantes, autosómicas recesivas o ligadas al cromosoma X. La forma hereditaria más común es la autosómica dominante. Estas formas hereditarias pueden presentar variabilidad intra o interfamiliar, incluso en sujetos que portan la misma mutación causal (Messina-Baas y Cuevas Covarrubias, 2017). Messina-Baas y Cuevas-Covarrubias han clasificado a las cataratas en 4 grupos de acuerdo a los genes asociados: a) genes implicados en cataratas sindromáticas, b) genes implicados en cataratas sindromáticas, pero con reportes sólo de catarata congénita, c) genes que sólo se asocian catarata, y d) genes cuyas mutaciones ocasionan catarata y otras anomalías oculares.

Catarata congénita por deficiencia de *GALK1*

La deficiencia de galactocinasa es una enfermedad autosómica recesiva muy rara que tiene una incidencia de 1 en un millón en Japón y 1 en 60000 nacidos en Estados unidos (Singh y cols., 2012) y que se caracteriza por la formación de catarata debido a la acumulación de galactitol en los cristalinos. Esas cataratas son bilaterales, son la única manifestación y aparecen durante las primeras semanas de vida, aunque ha sido reportado en recién nacidos (Scriver y cols., 2002). El diagnóstico de GALK1 se sospecha en niños con cataratas inexplicables e hiperglactosemia. Se comprueba con el test de la actividad de GALK1 (Beutler test) y por análisis molecular del gen *GALK1*. Las cataratas pueden ser

reversibles cuando se inicia una dieta libre de galactosa tempranamente, después de unos pocos meses de edad, los cambios ya son permanentes (Oberman y cols., 1972). A la fecha se han reportado cerca de 40 mutaciones, la mayoría de las cuales son sustituciones que causan mutaciones de sentido equivocado (http://www.hgmd.cf.ac.uk/ac/validate.php).

DISTROFIAS RETINIANAS HEREDITARIAS: RETINOSIS PIGMENTARIA

Las distrofias de la retina son un grupo de enfermedades genéticas caracterizadas por la degeneración y atrofia de capas o células retinianas específicas. Constituyen la causa más común de deficiencia visual hereditaria y tienen una prevalencia estimada de 1 en 1,500-2,000 sujetos en la población general (Berger y cols., 2010). Actualmente, se han identificado 261 genes relacionados a distrofias retinianas (https://sph.uth.edu/retnet/sum-dis.htm#D-graph), lo que convierte a esta enfermedad en una de las más heterogéneas genéticamente en el humano. Las distrofias retinianas hereditarias presentan una gran variación en sus características fenotípicas y esto puede deberse a mutaciones en diferentes genes, diferentes mutaciones en un mismo gen, variabilidad en el genoma en el que un gen mutante es expresado o a la modulación del fenotipo por factores ambientales. Además, el diagnóstico preciso puede dificultarse por los cambios retinianos que suceden durante la progresión de la enfermedad (Bird, 1995). En años recientes, las técnicas de análisis genético molecular han revolucionado la comprensión de las bases biológicas moleculares de las distrofias retinianas hereditarias (Gupta y cols., 2017).

Las distrofias retinianas se clasifican de acuerdo a su evolución clínica en estacionarias o progresivas y también de acuerdo al sistema de fotorreceptores (conos o bastones) que está principalmente afectado. Los conos operan durante condiciones de luz

brillante (fotópicas) y proporcionan la visión tricrómica en color, mientras que los bastones funcionan en la oscuridad (escotópica). La región macular localizada en el centro de la retina es rica en conos y se encarga de maximizar la visión detallada, en tanto que la región periférica es dominada por los bastones que proporcionan un campo de visión adecuado y detección de movimiento (Bessant y cols., 2001; Michaelides y cols., 2003).

Distrofias Retinianas de bastones o periféricas: Las alteraciones que afectan primariamente el sistema de bastones como la retinitis pigmentosa y la coroideremia se presentan inicialmente con ceguera nocturna (nictalopia) y pueden progresar hasta causar pérdida sustancial del campo visual periférico. Sin embargo, la visión central está preservada, al menos hasta etapas tardías de la enfermedad. Estas enfermedades se clasifican como distrofias periféricas (Bird, 1975; Wang y cols., 2005; Hims y cols., 2003; Hartong y cols., 2006; Hamel, 2006).

Distrofias retinianas de conos o centrales: Las alteraciones primarias del sistema de conos (distrofias de conos) se manifiestan inicialmente con pérdida de la agudeza visual central (visión borrosa) y de la visión al color y no afectan significativamente la visión periférica a menos que el sistema de bastones se encuentra también afectado (distrofias cono-bastón). Algunas enfermedades afectan a ambos sistemas de fotorreceptores en la zona especializada central de la retina, estas enfermedades son llamadas distrofias maculares (Michaelides y cols., 2003; Hamel, 2007; Voo y Small, 2004). Dentro de este grupo, la enfermedad de Stargardt o distrofia macular juvenil es la alteración más común.

Distrofias retinianas sindrómicas: Aunque la mayoría de pacientes con una distrofia retiniana tienen una degeneración retiniana sin asociación a anomalías extraoculares, existen algunas formas de distrofia retiniana sindrómicas en las que la degeneración retiniana progresiva se acompaña de alteraciones sistémicas. En algunos casos, estas formas

sindrómicas son causadas por defectos metabólicos conocidos; por lo tanto, permitiendo conocer las bases fisiopatológicas de la alteración retiniana. Durante la evaluación de un paciente con distrofia retiniana, el clínico debe estar alerta acerca de posibles signos fenotípicos acompañantes que permitan la identificación de entidades sindrómicas bien definidas. Tal es el caso de la hipoacusia en el síndrome de Usher, la polidactilia y obesidad en el síndrome de Bardet-Biedl, y la ptosis y oftalmoplegia progresiva en el síndrome de Kearns-Sayre, entre otros (Hims y cols., 2003; Hartong y cols., 2006; Reiners y cols., 2006; Kremer y cols., 2006; Zaghloul y Katsanis, 2009; López-Gallardo et al., 2009).

Retinosis Pigmentaria no sindromáticas

Las retinopatías hereditarias es una de las retinopatías causantes de ceguera y están caracterizadas por una degeneración lenta y progresiva de la retina (Coco y cols. 2009). La mayoría de los casos son debidos a mutaciones en un sólo gen. Esas mutaciones ocurren principalmente en los fotoreceptores (conos o bastones) y con menor frecuencia en el epitelio pigmentario de la retina (EPR). Las retinopatías hereditarias se clasifican de acuerdo al tipo de herencia (cuando es posible identificarlo) en autosómicas dominantes, autosómicas recesivas o ligadas al cromosoma X. De las formas más frecuentes y severas de retinopatías está la retinosis pigmentaria (RP).

La RP tiene una prevalencia que puede ir desde 1 en 750 a 9000 personas (Na y cols., 2017) y está caracterizada por depósitos de pigmentos llamadas espículas óseas, las cuales resultan de degeneración de los fotoreceptores, predominantemente de la periferia de la retina. Esta enfermedad es genética y fenotípicamente heterogénea con mutaciones que afectan entre el 0.025-0.04% de la población mundial (Soest y cols., 1999). La sintomatología puede estar restringida al ojo (RP no sindromática), pero un 20-30% de los

pacientes tiene alteraciones sistémicas (RP sindrómica), que a la fecha incluyen aproximadamente 30 distintos síndromes (Hartong y cols 2006).

En la mayoría de los casos, hay una degeneración inicial de bastones con subsecuente degeneración de los conos (Hamel, 2006). Como los bastones están concentrados en la periferia de la retina, las personas afectadas manifiestan de deterioro progresivo del campo visual periférico, hasta que finaliza como visión en túnel (Cuenca y cols., 2014). Según Hartong y cols. La enfermedad puede ser dividida en 3 etapas (Hartong y cols., 2006). En etapas iniciales que normalmente ocurren durante la adolescencia temprana, la nictalopía es el principal síntoma, la cual ocurre por disfunción de los bastones seguido por apoptosis y pérdida de los mismos. Esa sintomatología es percibida principalmente en lugares con luz tenue o escasa. En la etapa intermedia, la ceguera nocturna se hace evidente por las dificultades para conducir o caminar en la noche. El diagnóstico clínico es posible a través de la exploración del fondo de ojo que revela la presencia de depósitos de pigmento. Un examen de campos visuales usualmente muestra constricción del campo visual y a menudo visión en túnel. En las etapas avanzadas, los pacientes discapacidad en la visión durante el día como resultado de la pérdida de la visión periférica y central. La exploración oftalmológica revela depósitos de pigmentos dispersos, atenuación de vasos retinianos, atrofia del EPR y coriocapilares, y palidez del nervio óptico. El electroretinograma (ERG) se caracteriza por una reducción a ausencia de la onda y b en la fase escotópica (Hartong y cols., 2006).

Genética molecular de la RP

La base genética y las mutaciones que llevan al desarrollo de la RP son muy complejas y heterogéneas.Por ejemplo, mutaciones en ciertos genes pueden ocasionar diferentes tipos

de retinopatías hereditaria, tales como la enfermedad de Stargardt, la amaurosis congénita de Leber o también pueden estar asociadas a RP. De forma similar se ha reportado que genes que causan formas sindromáticas de RP, tales con el síndrome de Bardet-Biedl, también han sido identificados como causa de RP no sindromática (Berger y cols., 2010; Forsythe y Beales, 2013; Nash y cols., 2015). Todo esto sugiere mecanismos subyacentes similares y vías comunes, a pesar de las diferencias en las características clínicas. Actualmente, se han identificados cerca de 70 genes como causantes de RP (http://www.sph.uth.tmc.edu/retnet/). Los patrones hereditarios de la RP no sindrómica incluyen formas autosómicas dominantes (15-25%), autosómicas recesivas (5-20%), ligadas al cromosoma X (5-15%) y patrones desconocidos (40-50%) (Coco y cols, 2009; Fahim y cols., 1993a, Ferrari y cols., 2011; Hartong y cols 2006; lipinski y cols., 2013; Oishi y cols., 2014). Los genes con mayor frecuencia de mutación incluyen *RHO*, *RPRF*, *PRPH2*, *RP1*, *IMPDH1* y *PRPF8* para las formas autosómica dominantes; *USH2A*, *ABCA4*, *PDE6A*, *PDE6B* y *RPE65* para las formas recesivas; y *RPGR* para las formas ligadas al X. Las mutaciones del gen *RHO* son responsables del 20-30% de las formas autosómicas dominantes, el gen *USH2A* se asocia al 10-15% de las formas autosómicas recesivas y las mutaciones de *RPGR* explican la mayoría de los casos ligados al X. Mutaciones en estos últimos tres genes explican aproximadamente 30% de todos los casos de RP (Fahim y cols., 1993b; Harton y cols., 2006).

HERRAMIENTAS GENÓMICAS: MAPEO DE HOMOCIGOSIDAD CON MICROARREGLOS DE SNPs Y SECUENCIACIÓN DE SIGUIENTE GENERACIÓN DE REGIONES CODIFICANTES (SECUENCIACIÓN DE EXOMA)

Microarreglos y mapeo de homocigosidad

Se estima que hasta 10% de la población mundial practica las uniones consanguíneas.. La forma más común de uniones consanguíneas es la de parientes de tercer grado (primos primeros-primos hermanos), la cual es ampliamente practicada en países de Medio Oriente, Arabia Saudita, la India y Brasil, mientras las de segundo grado (tío-sobrina) está limitado a ciertas comunidades de países como la India donde este tipo de unión se considera legal (Khlat y Khoury, 1991). En tales poblaciones consanguíneas, el promedio poblacional de coeficiente de consanguinidad (porcentaje del genoma que es autocigoto) es considerablemente más alto que la población no consanguínea (Mueller y Bishop, 1993). En un autocigoma, que es el conjunto completo de intervalos homocigotos del genoma, un alto porcentaje se aproxima al coeficiente de consanguinidad (Alkuraya, 2010a). De esta forma, en sociedades en las cuales las uniones consanguíneas son comunes se eleva el tamaño de autocigomas cuando se compara con poblaciones no consanguíneas (Miano y cols. 2000). Mientras cada bloque autocigoto representa haplotipos que son idénticos a causa de que segregan desde un ancestro común (IBD: idéntico por descendencia), los bloques homocigotos pueden representar haplotipos que son idénticos, aunque surgen independientemente en la población (*identical by state*, IBS). Así, la evaluación de autocigosidad es un proceso de dos pasos que inicia por la determinación de homocigosidad seguido por diferenciación entre IBS de IBD. Cuando una población tiene

su origen en unos pocos fundadores, esos alelos asumirán una alta frecuencia, de modo que dos individuos de esa población podrían ser portadores y tendrían riesgo de tener un descendiente afectado (Alkuraya, 2013).

El uso de marcadores de DNA es esencial para la determinación de la homocigosidad. Históricamente, los microsatélites se utilizaron comúnmente para este proósito, pero estos han sido reemplazados por los SNPs, que son los polimorfismos más abundantes del genoma humano (en promedio existe un SNP cada kilobase). La utilización de microarreglos o "chips" de SNPs permite la identificación simultánea de miles de estos polimorfismos en un genoma bajo estudio (Strachan y Read, 2011). Dichos arreglos están disponibles comercialmente en plataformas de microarreglos diseñados por casas comerciales como Affymetrix o Agilent (Alkuraya, 2012).

En el mapeo de homocigosidad la distinción entre IBD e IBS es un proceso esencialmente probabilístico. Entre más grande sea un bloque de homocigosidad, es más probable que contenga SNPs que se originen de una región con baja recombinación y que representen LD (desequilibrio de ligamiento). Otro factor importante que se debe tomar en cuenta en la ancestría, factor que se correlaciona con el número de eventos de recombinación. Tomando en cuenta estos dos puntos, el valor de corte del tamaño de la región homocigota debe ser más grande cuando represente un IBD, y será más fuerte entre más cercano sea el grado de parentesco. Asumiendo que la mutación se encuentra en una posición Z. La probabilidad de recombinación en la posición Z-1Mb es alrededor del 1% y que similarmente la probabilidad de recombinación en la posición Z+1 es alrededor del 1%. Así, para la mutación la probabilidad de recombinación se encuentra dentro de un intervalo de 2 Mb (Alkuraya, 2012). Tomando en cuenta ese valor de corte en el mapeo de

homocigosidad, en la mayoría de estudios donde se analizan regiones < 2Mb rara vez se identifican mutaciones homocigotas.

En enfermedades autosómicas recesivas, los individuos afectados presentan mutaciones en ambas copias del mismo gen. En caso de que un individuo presente una mutación homocigota, la misma mutación es heredada de ambos padres. Asumiendo que la mutación en ambos padres se origina desde el mismo alelo ancestral, no sólo la mutación sino la región cromosómica alrededor de ella será idéntica en ambos cromosomas del paciente. Esta región, llamada Tl, es idéntica por descendencia (IBD). El tamaño de esa región cromosómica dependerá de la tasa de recombinación la cual a su vez depende del número de generaciones entre el ancestro portador de la variante y el paciente afectado (Woods y cols. 2006). Las regiones homocigotas presentes en afectados pueden ser detectadas por el uso de microarreglos que permiten caracterizar miles, incluso millones, de SNPs distribuidos en todo el genoma. Cuando un SNP identificado es idéntico en cada uno de los alelos, el SNP es llamado homocigoto. Cuando hay intervalos significantes de SNPs homocigotos consecutivos juntos componen regiones homocigotas, las cuales pueden suceder por azar y se consideran propiamente “homocigotas”, o pueden ser originadas de un mismo ancestro, denominándose autocigotas, como se comentó anteriormente (figura 1) (Littink y cols. 2012).

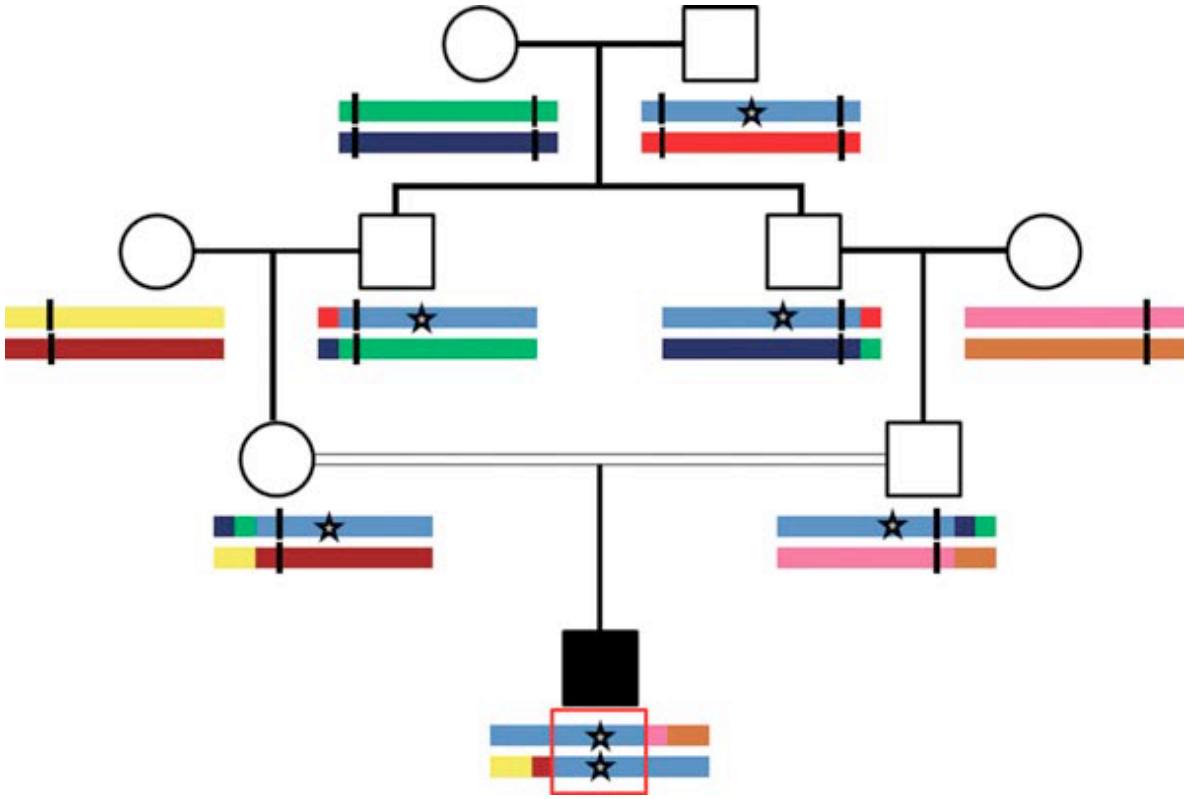

Figura 1. Árbol genealógico donde se muestra como un alelo ancestral (*) es segregado a través de las generaciones y como el haplotipo original de preserva parcialmente hasta última generación que hereda los dos alelos mutados (**). El haplotipo original va perdiendo regiones y eso se traza con diferentes colores. El haplotipo alrededor (muy cercano) a la mutación se preserva y es lo que representa las regiones homocigotas alrededor de las mutaciones que son el fundamento de esta técnica genómica. (Imagen tomada de: Alkuraya, 2012).

Secuenciación de siguiente generación: secuenciación de exoma

Las tecnologías NGS han revolucionado el diagnóstico molecular al permitir secuenciar simultáneamente miles de genes o incluso genomas completos en un único experimento (Katsanis y Katsanis., 2013; Strande y Berg, 2016). Estos métodos han demostrado una gran efectividad para identificar la causa subyacente de enfermedades

monogénicas raras o con un diagnóstico inespecífico, y han demostrado también gran eficacia en el diagnóstico de enfermedades con heterogeneidad genética en los diversos campos de la medicina clínica. Actualmente, diferentes técnicas de NGS pueden ser utilizadas para propósitos diagnósticos, incluyendo secuenciación completa del genoma (*whole genome sequecning*: WGS), secuenciación de los exones de todos los genes codificantes de proteínas (*whole exome sequencing*: WES) o la secuenciación dirigida específica de genes conocidos causantes de enfermedad (*targeted resequencing*: TRS) (de Koning y cols., 2015).

Proceso de secuenciación de exoma

En el proceso de secuenciación el DNA genómico es fragmentado por enzimas denominadas transposasas, las cuales también a la vez que fragmentan también añaden unas pequeñas secuencias llamadas adaptadores en cada extremo. A continuación se unen unas sondas de hibridación que son secuencias cortas de RNA acopladas a biotina que a su vez se adhieren a las regiones codificantes o intrónicas flanqueantes de todos los exones. Después estas regiones unidas a biotina son capturadas por perlas de estreptovidina por medio de un imán unido a estreptovidina. A continuación estos fragmentos son amplificados por PCR y están listos meterse a secuenciar.

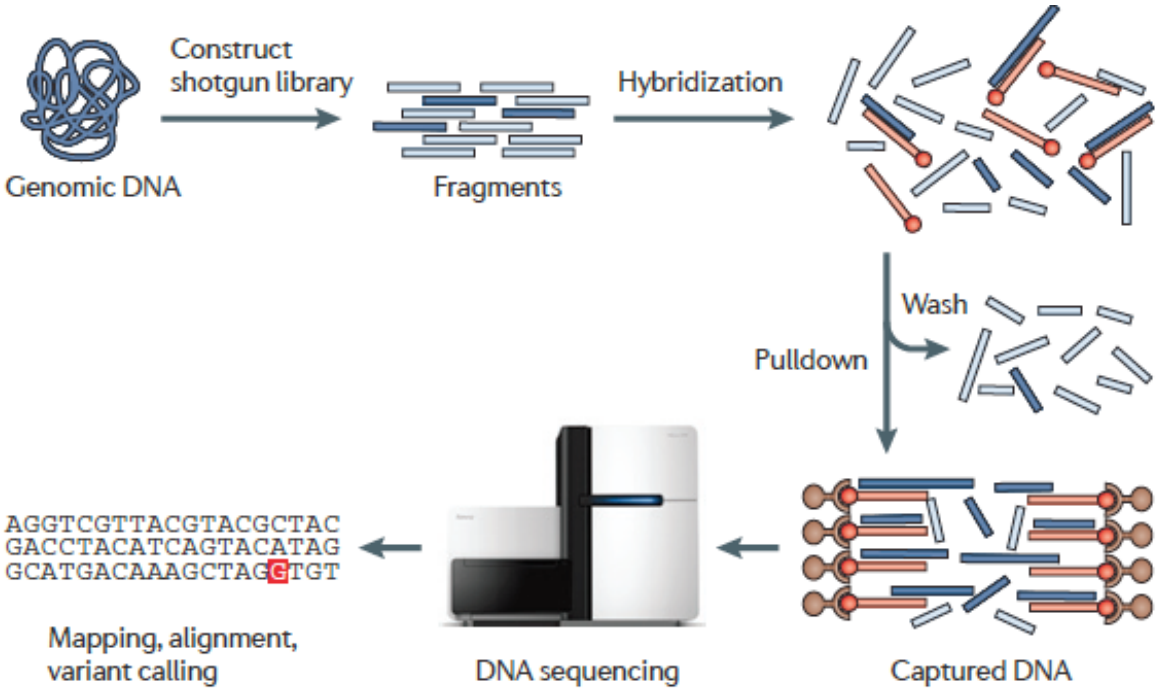

Figura 2. Proceso general de la secuenciación del exoma. Tomado de: Bamshad y cols., 2011).

a.Secuenciación de genoma completo (WGS)

Este método puede ser utilizado para determinar la secuencia completa del DNA, incluyendo regiones codificantes y no codificantes. La mayor ventaja es que la información genómica completa estará disponible de una forma relativamente rápida, y podrá ser utilizada para la identificación de variantes causales de enfermedad en caso de enfermedades monogénicas y también será de utilidad para la identificación de variantes de susceptibilidad para enfermedades complejas debido a que puede identificar variantes raras con frecuencias alélicas menores (MAF) <5% (Xuan y cols., 2013).

Una desventaja del WGS es que produce una gran cantidad de datos que requieren un filtrado extenso a fin de proveer datos significativos acerca de las variantes génicas relacionadas o causantes de la enfermedad. Otro problema es ético, y consiste en la

identificación de variantes patogénicas que no están relacionadas a la enfermedad original para la cual se realizó el estudio (de Koning y cols., 2015).

b.Secuenciación de exoma completo (WES)

Este abordaje puede analizar simultáneamente las regiones codificantes de todos los genes causantes de la enfermedad, como también para los genes potencialmente causantes de enfermedad, o incluso puede utilizarse para aquellas entidades donde no se conoce la causa genética de la enfermedad. Para realizar WES todo el DNA codificante necesita ser enriquecido. La mayor ventaja de la WES es que todos los genes están incluidos en el análisis, genes conocidos causantes de enfermedad y aquellos que aún no han sido relacionados a patologías. En particular, WES puede revelar genes nuevos para enfermedad o mutaciones que todavía no sido asociados con ciertos fenotipos. WES es la mejor técnica para la identificación de mutaciones de novo en un trío (padres e hijo) para enfermedades heterogéneas con un gran número de genes putativos (Vissers y cols., 2010).

No obstante, hay también algunos inconvenientes, como lo son que algunos exones no son incluidos, o la baja cobertura en ciertos exones, principalmente relacionados a secuencias ricas en G-C (Gilissen y cols., 2012).

C.Secuenciación dirigida (TRS)

Este abordaje es ampliamente utilizado como una estrategia para enriquecer y analizar las regiones codificantes de solamente los genes de interés para una enfermedad específica o alguna categoría diagnóstica. Es conocido también como secuenciación de paneles diagnósticos de enfermedades hereditarias (SureSelect [https://www.genomics.agilent.com/article.jsp?pageId=3083], TruSight One

[https://www.illumina.com/products/by-type/clinical-research-products/trusight-one.html]) (o para evaluar el riesgo de cáncer (Targeted cancer sequencing) (https://www.illumina.com/areas-of-interest/cancer/research/sequencing-methods/targeted-cancer-seq.html). Hay algunas ventajas de restringir el análisis mutacional a un número limitado de genes, como lo es una mayor profundidad de lectura con respecto a WGS y WES. Otra ventaja es un menor desafío en el análisis de datos e interpretación de las variantes (Sikkema-Raddatz y cols., 2013). Además, dependiendo de la plataforma y la estrategia de enriquecimiento utilizada, se pueden analizar varios cientos de genes de múltiples muestras de pacientes en una simple corrida y en un periodo de tiempo corto (de Koning y cols., 2015). Utilizamos dos paneles, *SureSelect inherited disease*, para el análisis de sólo 2742 genes que se conocen causan enfermedades hereditarias (https://www.genomics.agilent.com/en/SureSelect-DNA-Target-Enrichment-Baits-/SureSelect-Panels/?cid=AG-PT-124&tabId=AG-PR-1306&Nty=1&Ntx=mode+matchall&Ntk=BasicSearch&N=4294967292+4294967234+4294967294&type=baseSearch&No=0&Ntt=5190-4651). El otro panel utilizado frecuentemente corresponde a *TruSight One* para el análisis de 4800 genes asociados a enfermedad, el cual tiene la opción de extenderse a 6700 genes (https://www.illumina.com/products/by-type/clinical-research-products/trusight-one.html).

JUSTIFICACIÓN

La última década ha sido testigo de avances dramáticos en el desarrollo de nuevos métodos de análisis genómicos y de su aplicación en el campo de la investigación y el diagnóstico clínico. Una consecuencia de esta revolución tecnológica es la creciente identificación de variantes causales de enfermedades raras y la subsecuente identificación de vías biológicas que permitan el reconocimiento de mejores blancos terapéuticos. Debido a la heterogeneidad clínica y genética de muchas de estas patologías mendelianas, los nuevos abordajes genómicos buscan proporcionar a los enfermos un diagnóstico clínico correcto y rápido y evitar lo que se conoce como una odisea diagnóstica. En México no se han llevado a cabo estudios genómicos de enfermedades mendelianas oftalmológicas raras o con heterogeneidad genética que nos permitan conocer rápidamente las bases moleculares de estas enfermedades. A partir de este tipo de estudios es posible identificar nuevos genes o reconocer mecanismos mutacionales novedosos en genes conocidos. Además, el análisis genómico permite el diagnóstico clínico para entidades con fenotipos desconocidos, pero con patrones hereditarios claramente mendelianos. La identificación correcta del gen causante de la enfermedad establece de manera precisa el patrón hereditario de la enfermedad, lo que permite un adecuado asesoramiento genético al afectado y a su familia.

Otro punto importante es que se prevé que las técnicas genómicas que han mostrado ser de alto rendimiento, precisas, de bajo costo y de certeza diagnóstica, serán pronto pruebas diagnósticas abiertas en los sistemas de salud para pacientes con alguna enfermedad mendeliana rara o que presente una alta heterogeneidad clínica y genotípica.

PLANTEAMIENTO DEL PROBLEMA

Las enfermedades mendelianas son patologías raras individualmente, pero de manera grupal pueden afectar entre 2 y 10% de la población; por lo tanto, son responsables de una gran proporción de la morbi-mortalidad general. Actualmente se han descrito más de 6000 patologías con patrón hereditario mendeliano, de las cuales cerca de la mitad aún no tienen una etiología genética reconocida.

Los métodos tradicionales para la identificación de variantes patogénicas para estas enfermedades monogénicas, como lo son amplificación del DNA por reacción en cadena de la polimerasa y la secuenciación de DNA tipo Sanger, en muchos casos siguen siendo una opción diagnóstica, principalmente cuando se conocen sitios calientes dentro del gen de estudio o cuando existe una muy fuerte correlación genotipo-fenotipo que indique que una enfermedad es causada únicamente por variaciones en un solo gen. No obstante, para enfermedades muy raras con sospecha de herencia mendeliana o para aquellas enfermedades monogénicas con alta heterogeneidad genética, los métodos tradicionales no son la primera elección. En su lugar, nuevas tecnologías genómicas como el mapeo de homocigosidad o la secuenciación de siguiente generación deben ser aplicadas como primera opción para el diagnóstico molecular.

PREGUNTA DE INVESTIGACIÓN

¿Son eficaces las tecnologías de análisis genómico para el diagnóstico molecular de casos seleccionados de oftalmopatías hereditarias raras?

HIPÓTESIS DE TRABAJO

Los abordajes genómicos de mapeo por homocigosidad utilizando microarreglos de SNPs y de secuenciación de siguiente generación permitirán reconocer la causa genética de oftalmopatías hereditarias raras y/o con heterogeneidad genética

OBJETIVO GENERAL

- Identificar la causa genética de oftalmopatías hereditarias raras utilizando las nuevas tecnologías de análisis genómico.

OBJETIVOS ESPECÍFICOS

- Identificar los genes responsables y las variantes patogénicas causales en casos seleccionados de oftalmopatías hereditarias raras.
- Establecer el diagnóstico definitivo en casos seleccionados de oftalmopatías hereditarias raras.

DESCRIPCIÓN DE CASOS CLINICOS

CASO #1

Paciente femenino de 2 meses de edad referida a la consulta de Genética por dismorfias faciales. Es originaria del municipio de Amatenango del Valle, Chiapas, hija de padres sanos, no consanguíneos. La paciente es la sexta y última hermana y sus otros 5 hermanos son completamente sanos. La madre de la paciente tuvo valoración prenatal regular y niega exposición a teratógenos. La propósito nació por parto eutócico con datos somatométricos dentro de rango normal. Al nacimiento presentó telecanto y un exceso de tejido subcutáneo que se acumula en región naso-palpebral y se extiende a la frente y al labio superior. Esta tumoración es blanda y redondeada. Además, la paciente presenta colobomas bilaterales en ambos párpados entre los tercio medio e interno de los párpados. Con los datos clínicos identificados en la paciente se establece un diagnóstico de síndrome de lipoma-coloboma nasopalpebral. Esta enfermedad es una entidad rara que únicamente ha sido descrita en 5 ocasiones previamente, dos de las cuales corresponden a casos familiares con una herencia autosómica dominante. Este caso ilustra la importancia de la secuenciación de exoma completo para la identificación de genes nuevos para enfermedades raras con un claro patrón de herencia mendeliana

CASO #2

Femenino de 64 años de edad que fue revisada por el Servicio de Genética debido a ceguera de etiología desconocida. Es originaria de una población endogámica en la provincia de Alajuela, Costa Rica. Los padres son consanguíneos (primos de primer grado), tuvieron 8 hijos, de los cuales cuatro presentaron cuadros clínicos similares. Los afectados desarrollaron ceguera legal desde la infancia temprana y a pesar de que algunos tuvieron cirugía oftalmológica se refiere que ésta no mejoró de manera significativa la visión. Esta familia no tuvo un diagnóstico clínico de la enfermedad, y en la actualidad no se cuenta con notas clínicas ni expedientes hospitalarios que pudieran orientar hacia la causa de la deficiencia visual. Los afectados desean conocer que les ocasionó su ceguera, y los familiares sanos desean conocer si existe riesgo de que la enfermedad pueda repetirse en las siguientes generaciones. Este es un caso de ceguera de etiología desconocida con clara herencia autosómica recesiva, por lo cual se les ofrece un estudio de análisis genómico denominado mapeo de homocigosidad, para la búsqueda de regiones homocigotas dentro de la cual podría encontrarse el gen responsable de la enfermedad.

CASO #3

Se estudió una familia de 4 generaciones con diagnóstico de distrofia retiniana hereditaria. Los padres de los afectados son sanos, no consanguíneos, provienen de una comunidad probablemente endogámica y tuvieron 10 hijos, 5 de ellos afectados por el mismo padecimiento retiniano. En otra rama de la familia muy cercana hay un matrimonio consanguíneo entre primos hermanos los cuales tienen un hijo aparentemente con la misma enfermedad retiniana. A la exploración oftalmológica todos los sujetos presentaron características clínicas similares caracterizados por mala agudeza visual y fotofobia desde la primera década de vida. La fundoscopia demostró discos ópticos pálidos, estrechamiento de arteriolas, depósitos de pigmentos intraretinianos, atrofia severa del EPR y espículas óseas en la periferia. ERG esta abolido. Este es un caso de distrofia retiniana de etiología desconocida, pero con un carácter mendeliano autosómico recesivo. Por lo anterior se pensó como primera opción realizar un análisis genómico por mapeo de homocigosidad debido a la afección de hermanos y a la consanguinidad demostrada en la genealogía. Varios genes candidatos dentro de la región homocigotas fueron secuenciados, pero no fueron identificadas mutaciones. Debido al resultado negativo, y la gran heterogeneidad clínica y genética de las distrofias retinianas, se decidió realizar un análisis de secuenciación de exoma.

DISCUSIÓN

DISCUSIÓN CASO #1

El síndrome de coloboma-lipoma nasopalpebral es una entidad extremadamente rara descrita previamente en sólo 5 casos, dos de ellos en formas familiares con franca herencia autosómica dominante (Penchaszadeh y cols. 1982; Akarsu y Sayli, 1991) y 3 casos esporádicos (Bock-Kunz y cols., 2000; Moreira y cols., 2003; Babu y cols., 2011). La paciente descrita en este trabajo cumplió todos los criterios establecidos para el síndrome, incluyendo lipomas nasopalpebrales congénitos bilaterales, colobomas bilaterales en ambos párpados, frente amplia, pico de viuda, telecanto e hipoplasia maxilar. (Penchaszadeh y cols., 1982). Los estudios de tomografía computada con reconstrucción ósea y de tejidos blando faciales, se demostraron varios hallazgos importantes: Primero que la separación de cantos internos fue un telecanto y no un hipertelorismo, lo cual es importante para hacer el diagnóstico diferencial con otras entidades genética como es la displasia frontonasal (Sedano y Gorlin, 1988). También se demostró que la tumoración observada en la región nasopalpebral correspondía a tejido lipomatoso, lo cual fue confirmado con una biopsia. Por medio de ecografía ocular se demostró nanoftalmos bilateral, lo que significa un nuevo hallazgo clínico en la enfermedad (Chacon-Camacho y cols., 2013).

Esta enfermedad como pertenece al grupo de enfermedades raras y esporádicas, el estudio de secuenciación de exoma ofrece nuevas oportunidades para llegar a un diagnóstico molecular (Ku y cols., 2011).

Para la identificación de la causa genética de esta enfermedad utilizamos un abordaje de secuenciación de exoma completo que permitió identificar una variante patogénica en un gen denominado *ZDBF2*. Como se describe en la publicación anexa, la

variante cumplió con criterios que permiten clasificarla como patogénica, entre otros ser una variante de novo, con efecto de truncamiento en la proteína, no está presente en bases de datos poblacionales y afecta a un gen localizado en una región de impronta probablemente implicado en el desarrollo craneofacial (Chacón-Camacho y cols., 2016). La mutación identificada en *ZDBF2* en nuestro caso de síndrome de lipoma-coloboma nasopalpebral, c.6245_624insTT (p.His2082fs*67) en estado heterocigota, provoca un desplazamiento del marco de lectura y predice una mutación terminadora 67 aminoácidos corriente abajo. Según las guías del Colegio Americano de Genetica Médica y Genómica la variante tendría un criterio muy fuerte (mutación que provoca un codón de paro y proteína de terminación prematura) y un criterio fuerte (mutación de novo, con ambos padres confirmados, en un paciente con la enfermedad y sin historia familiar) lo cual correspondería a una variante patogénica (Richards y col., 2015). A pesar de que se conocen muy pocos datos acerca de la función del gen, se propone que podría intervenir en la morfogénesis del desarrollo medio-superior de la cara, y que su defecto provoca una hiperplasia del tejido adiposo dirigiendo al desarrollo de los lipomas característicos de la enfermedad (Penchaszadeth y cols., 1982). Conociendo otros genes improntados, como los identificados en el síndrome de Beckwith Widemann por ejemplo (Shuman y cols., 2016), que es una enfermedad también caracterizada por aparición de tumor embrionarios como tumor de Wilms o hepatoblastoma, proponemos que está región improntada donde se encuentra el gen *ZDBF*, podría tener funciones similares que regulen el sobrecremiento de ciertas estirpes celulares, como el tejido adiposo.

Para concluir es importante mencionar, que en otro grupo de estudio internacional ya se identificó un paciente en quien se realizó estudio genético del gen *ZDBF2*, con

resultado positivo para una variante patogénica (comunicación personal), lo cual validaría de forma definitiva al gen como causante del síndrome lipoma-coloboma nasopalpebral.

De forma similar, a partir del año 2010 en muchas enfermedades raras y esporádicas con la utilización de secuenciación de exoma completo se han identificado los genes causantes de enfermedades, como por ejemplo el gen *MLL2* para el síndrome de Kabuki (Ng y cols., 2010) y el gen SETBP para el síndrome de Schinzel-Giedion (Hoischen y cols., 2010).

DISCUSIÓN CASO #2

Muchos pacientes con enfermedades genéticas, como en la familia descrita no cuentan con un diagnóstico específico. La práctica estándar de la medicina involucra el reconocimiento de las características clínicas-fenotípicas específicas, además de las características derivadas de estudios de laboratorio y gabinete, histopatológicos y genéticos. Todos estos hallazgos juntos tienen el propósito de generar un diagnóstico al paciente. Pese a ello, muchos pacientes permanecen sin un diagnóstico. Nuestro caso clínico tiene una etiología oscura ya que se carecen de antecedentes que guíen hacia un probable diagnóstico oftalmológico-genético. El antecedente de que los padres son consanguíneos y que existen 4 hermanos afectados, sugiere fuertemente una entidad con patrón hereditario autosómico recesivo. A pesar de que la enfermedad está limitada a los ojos y no hay afección sistémica ni alteración de la capacidad intelectual de los sujetos enfermos, la lista para enfermedades recesivas que causan ceguera es amplia, e incluyen glaucoma congénito, distrofia corneal congénita, distrofias retinianas de inicio temprano, y cataratas congénitas, entre otras. Los genes candidatos para estas entidades pueden representar decenas de genes y es por lo tanto

impráctico intentar realizar un diagnóstico con las técnicas moleculares tradicionales de PCR y secuenciación Sanger. La familia deseaba conocer la causa de la ceguera y esto es importante por varias razones: a) para instaurar un tratamiento potencial, principalmente en las fases iniciales de la enfermedad; b) para dar un riesgo de recurrencia a los afectados y a los familiares sanos; c) para proveer un pronóstico. Un largo periodo para que se realice un diagnóstico genético es actualmente referido como una "odisea diagnóstica" y es lo que está familia ha padecido por muchos años (Yang y cols., 2013). La técnica de análisis genómico como el mapeo de homocigosidad es una herramienta valiosa y costo-efectiva y es una alternativa para el establecimiento de las bases genéticas de la enfermedad, principalmente cuando se trata de familias con consanguinidad y se tienen que identificar regiones homocigotas (Gillespie y cols., 2014). Utilizando un microarreglo que contiene aproximadamente 250 mil SNPs se identificaron 2 regiones homocigotas, una en el cromosoma 3 y otra en el cromosoma 17. Esta última región homocigota era de una extensión de 6.6 Mb (mega bases) y dentro de ella se encontraron 143 genes, uno de los cuales era un gen conocido asociado a catarata, sin afección sistémica: *GALK1*. El análisis dirigido por secuencia Sanger identificó la mutación c.1144C>T (p.Q382*), que predice una proteína de terminación prematura. Para demostrar que la variante fuera patogénica se realizó el análisis de segregación familiar que demostró que todos los hermanos afectados presentaban la misma variante en estado homocigoto, mientras que los sanos tuvieron únicamente la variante silvestre. Con este estudio la familia pudo por fin conocer cuál era su diagnóstico y se pudo realizar una adecuada asesoría genética para toda la familia. La deficiencia de la enzima galactosa 1 fosfato (GALK1) es una entidad muy rara, que origina catarata congénita, la cual es reversible cuando se instaura de manera rápida la suspensión de la galactosa en la dieta (Fridovich-Keil, y cols., 2008). Se debe mencionar que la

mutación identificada en estos pacientes fue reportada anteriormente por Kolosha y cols., y de manera interesante los afectados con la misma mutación en ese estudio eran originarios de Costa Rica, lo que indica un posible efecto de mutación fundadora en esa población.

DISCUSIÓN CASO 3

La heterogeneidad de locus, definida como la existencia de muchos loci asociados a la misma enfermedad, es una característica de numerosas entidades mendelianas (Nausbaum y cols., 2015). Un ejemplo claro lo representan las distrofias retinianas, para las que se han identificado aproximadamente 300 loci relacionados y se han caracterizado cerca de 260 genes causales. Mutaciones en al menos 60 de estos genes ocasionan retinosis pigmentaria no sindromática con diversos patrones de herencia (https://sph.uth.edu/retnet/sum-dis.htm#D-graph). Clásicamente, la elección del gen a analizar para realizar diagnóstico molecular en enfermedades con heterogeneidad genética depende de varios criterios que incluyen: a) iniciar con el gen más frecuentemente mutado, b) identificar alguna correlación genotipo-fenotipo, y c) el conocimiento previo de un efecto fundador de la mutación en un grupo étnico estudiado (Koenekoop y cols., 2007). Si la mutación causal no es identificada por los criterios previamente mencionados, el análisis puede ser extendido a otros genes. Pero esta solución del análisis gen por gen es desgastante en cuanto a tiempo de procesamiento y de costo económico. El advenimiento de las tecnologías de análisis genómico ha facilitado de una manera sin precedente el diagnóstico molecular en enfermedades con heterogeneidad genética cono las distrofias retinianas.

Los resultados obtenidos en el caso familiar de distrofia retiniana presentado en este trabajo demuestran la utilidad del abordaje de exoma completo en el diagnóstico molecular.

Los afectados mostraban daño severo del área macular y de la retina periférica, característica que impedía una clasificación clínica correcta. Debido a la heterogeneidad genética propia de este grupo de enfermedades y considerando que las mutaciones del gen *ABCA4* son la causa más frecuente de distrofias maculares (Allikmets y cols., 1997) y de distrofias de cono-bastón (Maugeri y cols., 2000), el primer abordaje fue la secuenciación Sanger de los 50 exones codificantes de este gen. Sin embargo, no se identificaron variantes patogénicas en *ABCA4* que explicaran el fenotipo en la familia. Posteriormente, tomando en cuenta que la genealogía sugería fuertemente una herencia autosómica recesiva, se procedió a realizar un mapeo de homocigosidad de genoma completo mediante microarreglos de SNPs. La hipótesis fue que la enfermedad era causada por una variante homocigota, idéntica por descendencia, que podía ser localizada en alguna región de autocigosidad demostrada en el genoma de los enfermos. Este abordaje ha sido utilizado con éxito en numerosos casos de retinosis pigmentaria autosómicas recesivas descritos en la literatura (Bandah-Rozenfeld y cols., 2010; Collin y cols., 2011). De este modo, el análisis genómico permitió identificar una región homocigota de aproximadamente 3 Mb en el cromosoma 18p11. Sin embargo, ninguno de los genes localizados dentro de tal región tenía asociación con enfermedades retinianas hereditarias, por lo que no se identificó un gen candidato. Uno de los genes dentro de la región fue *TUBB6* (tubulina beta 6) que, aunque no ha sido asociado a enfermedad en humanos, se expresa abundantemente en retina. Se procedió a secuenciar este gen en DNA de uno de los afectados y se demostró que no presentaba variantes patogénicas.

De acuerdo a estos resultados, se decidió realizar secuenciación de exoma completo para la identificación de la causa de la enfermedad en esta familia, teniendo como posibilidades la mutación en un gen nuevo o incluso la presencia de heterocigocidad

compuesta (Littink y cols., 2012). Como se describe en el artículo anexo, la secuenciación de exoma completo permitió el reconocimiento de dos variantes patogénicas heterocigotas en el gen *RDH12*, c.295C>A (p.L99I) y c.446T>C (p.L149P) en DNA de los afectados, es decer, mutaciones en estado heterocigoto compuesto. El gen RDH12 se expresa ampliamente en los fotorreceptores y tiene un papel fundamental en la conversión del todo-transretinal a 11 cis-retinal en el ciclo de la vitamina A (Haeseller y cols., 2002). A la fecha se han identificado cerca de 100 mutaciones en RDH12 causantes de distrofias retinianas severas, particularmente amaurosis congénita de Leber y retinosis pigmentaria de inicio temprano (Benayouny cols., 2009; Li y cols., 2017). La mayoría de las variantes patogénicas descritas en RDH12 son sustituciones que predicen una mutación de sentido alterado en la proteína. Las mutaciones de RDH12 explican del 3 al 7% de los casos autosómicos recesivos de distrofias retinianas (Valverde, 2009). Interesantemente, las mutaciones heterocigotas compuestas, como la identificada en la familia, no son infrecuentes en este gen (Li et al., 2017; Benayoun y cols., 2009; Valverde y cols., 2009). La presencia de 2 alelos patogénicos distintos en esta familia explica la razón por la que el análisis de homocigosidad no fue concluyente y ejemplifica la importancia de considerar esta posibilidad incluso en casos de enfermedades genéticas en los que exista consanguinidad entre los padres o practicas endogámicas. A la fecha no existe una correlación genotipo-fenotipo en este gen, aunque se considera que sus mutaciones resultan en formas muy severas de distrofias retinianas (Chacon-Camacho y cols., 2013; Gong y cols., 2015).

CONCLUSIONES

- Las tecnologías genómicas fueron eficaces para llegar a un diagnóstico molecular en los tres casos estudiados.
- Se estableció un diagnóstico clínico en las 2 entidades con etiología oscuras y odisea diagnóstica: ceguera secundaria a catarata congénita (mutaciones en *GALK1*) y retinopatía mendeliana (retinosis pigmentaria por mutaciones en *RDH12*).
- Se identificó un gen causal (*ZDBF2*) para el síndrome de lipoma-coloboma nasopalpebral
- Se identificó una variante patogénica nueva en el gen *RDH12.*
- Se identificó una mutación en el gen GALK1 causante de ceguera en la familia costarricense.

Referencias

Akarsu AN, Sayli BS. Nasopalpebral lipoma-coloboma syndrome. Clin Genet 1991; 40: 342–344.

Alkuraya FS. Autozygome decoded. Genet Med 2010; 12: 765-71.

Alkuraya FS. Discovery of rare homozygous mutations from studies of consanguineous pedigrees. Curr Protoc Hum Genet 2012; Chapter 6: Unif6.12.

Alkuraya FS. The application of next-generation sequencing in the autozygosity mapping of human recessive diseases. Hum Genet 2013; 132: 1197-211.

Allikmets R, Shroyer NF, Singh N, Seddon JM, Lewis RA, Bernstein PS, Peiffer A, Zabriskie NA, Li Y, Hutchinson A, Dean M, Lupski JR, Leppert M. Science 1997; 277: 1805-7.

Babu NS, Raviprakash D, Kumar R. Nasopalpebral lipoma coloboma syndrome. Indian J Ophthalmol 2001; 59: 379–80.

Baired PA, Anderson TW, Newcombe HB, Lowry RB. Genetic disorders in children and Young adults: a population study. Am J Hum Genet 1998; 42: 677-93.

Bakker E. Is the DNA sequence the gold standard in genetic testing? Quality of molecular genetic test assessed. Clin Chem 2006; 52: 557-8.

Bamshad MJ, Ng SB, Bigham AW, Tabor HK, Emond MJ, Nickerson DA, Shendure J. Exome sequencing as a tool for Mendelian disease gene discovery. Nat Rev Genet 2011: 12: 745-55.

Bandah-Rozenfeld D, Mizrahi-Meissonnier L, Farthy C, Obolens A, Chowers I, Peér J, Merin S, Ben-Yosef T, Ashery-Padan R, Banin E, Sharon D. Homozygosity mapping reveals null mutations in FAM161A as a cause of autosomal-recessive retinitis pigmentosa. Am J Hum Genet 2010; 87: 382-91.

Benayoun L, Spiegel R, Auslender N, Abbasi AH, Rizel L, Hujeirat Y, Salama I, Garzozi HJ, Allon Shalev S, Ben-Yosef T. Genetic heterogeneity in two consanguineous families segregating early onset retinal degeneration: the pitfalls of homozygosity mapping. Am J Med Genet A 2009; 149A: 650-6.

Benneth JT, Tan TY, Alcantara D, Tétrault M, Timms AE, Jensen D, Collins S, Nowaczyk MJM, Lindhurst MJ, Christensen KM, Braddock SR, Brandling-Bennett H, Hennekam RCM, Chung B, Lehman A, Su J, Ng S, Amor DJ; University of Washington Center for Mendelian Genomics; Care4Rare Canada Consortium, Majewski J, Bisecker LG, Boycott KM, Dobyns, O¨Driscoll M, Moog U, McDonell LM. Mosaic activing mutations in FGFR1 cause Encephalocraniocutaneous lipomatosis. Am J Hum Genet 2016; 98: 579-87.

Berger W, Kloeckener-Gruissem B, Neidhardt J. The molecular basis of human retinal and vitreoretinal diseases. Prog Retin Eye Res 2010; 29: 335-75.

Bird AC. X-linked retinitis pigmentosa. Br J Ophthalmol 1975; 59: 177-99.

Bird HC. Retinal photoreceptor dystrophies (51st Edward Jackson Memorial Lecture). Am J Ophthalmol. 1995; 119: 543-62.

Bock-Kunz AL, Lyon DB, Singhal VK, Grin TR. 2000. Nasopalpebral lipoma-coloboma. Arch Ophthalmol 200; 118: 1699–701.

Boppudi S, Bögershausen N, Hove HB, Percin EF, Aslan D, Dvorsky R, Kayhan G, Li Y, Cursiefen C, Tantcheva-Poor I, Toft PB, Bartsch O, Lissewski C, Wieland I, Jakubiczka S, Wollnik B, Ahmadian MR, Heindl LM, Zenker M. Specific mosaic KRAS mutations affecting codón 146 cause oculoectodermal síndrome and encephalocranious lipomatosis. Clin Genet 2016; 90: 334-42.

Botstein D, Risch N. Discovering genotypes underlying human phenotypes: past success for mendelian disease, future, approach for complex disease. Nat Genet 2003; 33: 228-37.

Cassidy SB, Allanson JE. (2010). Introduction. En Management of genetic syndromes (4). USA, Canada: Wiley.

Chacon-Camacho OF, Jitskii S, Buentello-Volante B, Quevedo-Martinez J, Zentneo JC. Exome identifies RDH12 compund heterozygous mutations in a family with severe retinitis pigmentosa. Gene 2013; 528: 178-82.

Chacon-Camacho OF, Lopez-Martinez MS, Vázquez J, Nava-Castañeda A, Martin-Biasotti F, Piña-Aguilar RE, Iñiguez-Soto M, Acosta-García J, Zenteno JC. Nasopalpebral lipoma-coloboma syndrome: clinical, radiological, and histopathological description of a novel sporadic case. Am J Med Genet 2013; 161A: 1470-4.

Chacon-Camacho OF, Sobreira N, You J, Piña-Aguilar RE, Villegas-Ruiz V, Zenteno JC. Exome sequencing identifies a de novo frameshift mutation in the imprinted gene ZDBF2 in a sporadic patient with Nasopalpebral Lipoma-coloboma syndrome. Am J Med Genet A 2016; 170: 1934-7.

Chacon-Camacho OF, Zenker M, Schanze D, Ledesma-Gil J, Zenteno JC. Novel F<rem1 mutations in a patient with MOTA síndrome: clinical findings, mutation update and review of FREM1-related disorders literatura. Eur J Med Genet 2017; 60: 190-94.

Coco M, Han SW, Sallum JM. Gene therapy for inherited retinal dystrophies. Arq Bras Oftalmol 2009; 72: 560-566.

Collin RW, van den Born LI, Klevering BJ, de Castro-Miró M, Littlink KW, Arimadyo K, Azam M, Yazar V, Zonneveld MN, Paun CC, Siemiatkowska AM, Strom TM, Hehr-Kwa JY, de Faber JT, van Schooneveld MJ, Heckenlively JR, Hoyng CB, den Hollander AI, Cremers FP. Hihg-resolution homozygosity mapping is a powerful tool to detect novel mutations causative of autosomal recessive RP in the Dutch population. Invest Ophthalmol Vis Sci 2011; 52: 2227-39.

Cuenca N, Fernandez-Sanchez L, Campello L, Maneu V, de la Villa P, Lax P, Pinilla I. Cellular responses following retinal injuries and therapeutic approach for neurodegenerative diseases. Prog Retin Eye Res 2014; 43: 17-75.

De Koning TJ, Jongbloed JD, Sikkema-Raddatz B, sinke RJ. Targeted next-generation sequencing panels for monogenetic disorders in clinical diagnostics: the opportunities and challenges. Expert Rev Mol Diagn 2015; 15: 61-70.

De Maria B, Mazzanti L, Roche N, Hennekam RC. Barber-Say síndrome and ablepharon-macrostomia síndrome: an overview. Am J Med Genet A 2016; 170: 1989-2001.

Dolfus H, Verloes A. Dysmorphology and orbital región: a practical clinical approach. Surv Ophthalmol 2004; 49: 547-61.

Fahim AT, Daiger SP, Weleber RG. Retinitis pigmentosa overview in: Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A, Bean LJH, Bird TD, Fong CT, Mefford HC, Smith RJH, Spephens K (eds), Genereview 1993b, Seattle, USA.

Ferrari S, Di Iorio E, Barbaro V, Ponzin D, Sorrentino FS, Parmeggiani F. retinitis pigmentosa: genes and disease mechanism. Curr Genomics 2011; 12: 238-249.

Forsythe E, Beales PL. Bardet-Biedl síndrome. Eur J Hum Genet 2013; 21: 8-13.

Friend SH, Bernards R, Rogelj S, Weinberg RA, Rapaport JM, Albert DM, Dryja TP. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. Nature 1986; 323: 643-46.

Fridovich-Keil, J.L.,Walter, J.H., 2008. Galactosemia. In: Scriver, Childs, Sly, Valle, Beaudet, Vogelstein, Kinzier, Antonarakis, Ballabio (Eds.), The On Line Metabolic and Molecular Bases of Inherited Disease, 72, pp. 1–92 (Accessed August 2013.Availablefrom: http://www.ommbid.com/OMMBID/the_online_metabolic_and_molecular_bases_of_ inherited_disease/b/abstract/part7/ch72).

Gad A, Laurino M, Maravilla KR, Matsushita M, Raskind WH. Sensorineural deafness, distinctive facial features, and abnormal cranial bones: a new variant of Waardenburg syndrome? Am J Med Genet A 2008; 146A: 18880-5.

Gilissen C, Hoischen A, Brunner HG, Veltman JA. Disease gene identification strategies for exome sequencing. Eur J Hum Genet 2012; 20: 490-7.

Gillespie RL, O`Sullivan J, Ashworth J, Bhaskar S, Williams S, Biswas S, Kenhdi E, Ramsden SC, Clayton-Smith J, Black GC, Lloyd IC. Personalized diagnosis and management of congenital caract by next-generation sequencing. Ophthamol 2014; 121: 2124-37.

Gillespie RL, Lloyd IC, Black GC. The use of autozygosity mapping and next-generation sequencing in undertanding anterior segment defects caused by an abnormal development of the lens. Hum Hered 2014; 77: 118-37.

Gong B, Wei B, Huang L, Hao J, Li X, Yang Y, Zhou Y, Hao F, Cui Z, Zhang D, Wang L, Zhang H. Exome sequencing identified a recessive RDH12 mutation in a family with severe early-onset retinitis pigmentosa. J Ophthalmol 2015 (epub).

Groesser L, Herschberger E, Ruetten A, Ruivenkamp C, Lopriore E, Zutt M, Langmann T, Singer S, Klingseisen L, Schneider-Brachert W, Toll A, Real FX, Landthaler M, Hafner C. Poszygotic HRAS and KRAS mutations cause nevus sebaceous ans Schimmelpenning síndrome. Nat Genet 2012; 44: 783-7.

Guercio JR, Martyn LJ. Congenital malformations of the eye and orbit. Otolaryngol Clin N Am 2007; 40: 113-140.

Gupta S, Chatterjee S, Mukherjee A, Mutsuddi M. Whole exome sequencing: uncovering causal genetic variants for ocular diseases. Exp Eye Res 2017; 164: 139-150.

Haeselee F, Jang GF, Imanishi Y, Driessen CAGG, Matsumura M, nelson PS, Palczewski K. Dual-substrate specific short chain retinol dehydrogenases from the vertebrate retina. J Biol Chem 2001; 277: 45537-46.

Hamel C. Retinitis pigmentosa. Orphanet J Rare Dis. 2006; 1:40.

Hamel CP. Cone rod dystrophies. Orphanet J Rare Dis 2007; 2:7.

Hartong D, Berson EL, Dryja TP. Retinitis pigmentosa. Lancet 2006; 368: 1795-809.

Harville HM, Held S, Diáz-font A, Davis EE, Diplas BH, Lewis RA, Brochowitz ZU, Zhou W, Chaki M, MacDonald J, Kayserili H, Beales PL, Katsanis N, Otto E, Hildebrandt F. Identification of 11 novel muatations in eight BBS genes by high resolution homozygosity mapping. Nat Rev Genet 2010; 47: 262-7.

Hims MM, Diager SP, Inglehearn CF. Retinitis pigmentosa: genes, proteins and prospects. Dev Ophthalmol 2003; 37:109-25.

Hoefele J, Wilhelm C, Sciesser M, Mack R, Heinrich U, Weber LT, Biskup S, Daumer-Haas C, Klein HG, Rost I. Expanding the mutation spectrum fro Fraser síndrome: identification of a novel heterozygous deletion in FRAS1. Gene 2013; 520: 194-7.

International Human Genome Sequencing Consortium. Finishing the euchromatic sequence of human genome. Nature 2004; 431: 931-45.

Hoischen A, van Bon BW, Gilissen C, Arts P, van Lier B, Steehouwer M, de Vries P, de Reuver R, Wieskamp N, Mortier G, Devriendt K, Amorim MZ, Revencu N, Kidd A, Barbosa M, Turner A, Smith J, Oley C, Henderson A, Hayes IM, Thompson EM, Brunner HG, de Vries BB, Veltman JA. De novo mutations of SETBP1 cause Schinzel-Giedion syndrome. Nat Genet 42: 483-85.

Khan AO. Microcornea with myopic chorioretinal atrophy, telecanthus and posteriorly-rotated ears: a distinct clinical síndrome. Ophthalmic Genet 2012; 33: 196-9.

Katsanis SH, Katsanis N. Molecular genetic testing and future of clinical genomics. Nat Rev Genet 2013; 14: 415-26.

Kaufman RL, Rimoin DL, Prensky AL, Sly WS. An oculocerebrofacial syndorme. Birth Defects Orig Artic Ser

Khlat M, Khoury M. Inbreeding and diseases: demographic, genetic, and epidemiologic perspectives. Epidemiol Rev 1991; 13: 28-41.

King RA, Rotter JI, Motulsky. (2001). Approach to genetic basis of common diseases. En The genetic basis of common diseases (8). USA: Oxford.

Koenekoop RK, Lopez I, den Hollander AI, et al. Genetic testing for retinal dystrophies and dysfunctions: benefits, dilemas and solutions. Clin Exp Ophthalmol 2007; 35: 473-485.

Kolosha V et a. Novel mutations in 13 probands with galactosemia deficiency. Hum Mut 2000; 15: 447-53.

Kremer H, van Wijk E, Märker T, Wolfrum U, Roepman R. Usher syndrome: molecular links of pathogenesis, proteins and pathways. Hum Mol Genet. 2006; 15 Spec No 2: R262-70.

Ku CS, Naidoo N, Pawitan Y. Revisiting mendelian disorders through exome sequencing. Hum Genet 2011; 129: 351-70.

Li A, Jiao X, Munier FL, Schorderet DF, Yao W, Iwata F, Hyakawa M, Kanai A, Chen MS, Lewis RA, Heckenlively J, Weleber RG, Traboulsi EI, Zhang Q, Xiao X, Kaiser-Kupfer M, Sergeev YV, Hejtmancik JF. Bietti crystalline corneoretinal dystrophy is caused by mutations in the novel gene CYP4V2. Am J Hum Genet 2004; 74: 817-26.

Levin AV. Congenital eye anomalies. Pediatr Clin North Am 2003; 50: 55-76.

Li Y, Pan Q, Gu YS. Phenotype-genotype correlation with Sanger sequencing identified retinol dehydrogensase 12 (RDH12 compound heterozygous varints in a Chinese family with Leber congenital amaurosis. J Zhejiang Univ Sci B 2017; 18: 421-429.

Lipinski DM, Barnard AR, Singh MS, Martin C, Lee EJ, Davies WI, MacLaren RE. CNTF gene therapy confers lifelong neuroprotection in a mouse model of human retinitis pigmentosa. Mol Ther 2015; 23: 1308-19.

Littink KW, den Hollander AI, Cremers FP, Collin RW. The power of homozygosity mapping: Discovery of new genetic defects in patients with retinal dystrophy. Adv Exp Med Biol 2012; 723: 345-51.

López-Gallardo E, López-Pérez MJ, Montoya J, Ruiz-Pesini E CPEO and KSS differ in the percentage and location of the mtDNA deletion. Mitochondrion. 2009; 9: 314-7.

Marchegiani S, Davis T, Tessadori F, van Haaften G, Brancati F, Hoischen A, Huang H, Valkanas E, Pusey B, Schanze D, Venselaar H, Vulto-van Silfhout AT, Wolfe LA, Tifft CJ, Zerfas PM, Zambruno G, Kariminejad A, Sabbagh-Kermani F, Lee J, Tsokos MG, Lee CC, Ferraz V, da Silva EM, Stevens CA, Roche N, Bartsch O, Farndon P, Bermejo-Sanchez E, Brooks BP, Maduro V, Dallapiccola B, Ramos FJ, Chung HY, Le Caignec C, Martins F, Jacyk WK, Mazzanti L, Brunner HG, Bakkers J, Lin S, Malicdan MC, Boerkoel CF, Gahl WA, de Vries BB, van Haelst MM, Zenker M, Markello TC. Recurrent mutations in the basic domain of TWIST2 cause ablepharon macrostomia and Barber-Say síndromes. Am J Hum Genet 2015; 97: 99-110.

Marlhens F, Bareil C, Griffoin JM, Zrenner E, Amalric P, Eliaou C, Liu SY, Harris E, Redmond TM, Arnaud B, Claustres M, Hamel CP. Mutations in RPE65 cause Leber´s congenital amaurosis. Nature Genet 1997; 17: 139-41.

Maugeri A, Klevering BJ, Rohrscheneider K, Blankenagel A, Brunner HG, Deutman AF, Hoyng CB, Cremers FP. Mutations in the ABCA4 (ABCR) gene are the major cause of autosomal recessive cone-rod dystrophy. Am J Hum Genet 2000; 67: 960-6.

Messina-Baas O, Cuevas-Covarrubias SA. Inherited congenital cataract: a guide to suspect the genetic etiology in the cataract genesis. Mol Syndromol 2017; 8: 58-78.

Miano MG, Jacobson SG, Carothers A, Hanson L, Teague P, Lovell J, Cideciyan AV, Haider N, Stone EM, Sheffield VC, Wright AF. Pitfalls in homozygosity mapping. Am J Hum Genet 2000; 67: 13-48-51.

Michaelides M, Hunt M, Moore AT. The genetics of inherited macular dystrophies. J Med Genet 2003; 40: 641-650.

Miller MT, Deutsch TA, Cronin C, et al. Amniotic bands as a cause of ocular anomalies. Am J Ophthalmol 1987; 104: 270-9.

Moreira Gonzalez A, Jackson IT. Surgical correction of the nasopalpebral lipoma-coloboma syndrome. Eur J Plast Surg 2003; 26: 263–7.

Mueller RF, Bishop DT. Autozygosity mapping, complex consanguinity, and autosomal recessive disorders. J Med Genet 1993; 30: 798-799.

Munier Fl, Korvatska E, Djemai A, Le Paslier D, Zografos L, Pescia G, Schorderet DF. Kerato-epithelin mutations in four 5q31-linked corneal dystrophies. Nature Genet 1997; 15: 247-251.

Na KH, Kim HJ, Kim KH, Han S, Kim P, Hann HJ, Ahn HS. Prevalence, age at diagnosis, mortality and cause of death in retinitis pigmentosa in Korea-A Nationwide population – based study. Am J Ophthalmol 2017; 176: 157-165.

Nash BM, Wright DC, Grigg JR

Nathans J, Hogness DS. Isolation and nucleotide sequence of the gene encoding human rhodopsin. Proc Nat Acad Sci 1984; 81: 4851-55.

Ng SB, Bigham AW, Buckinham KJ, Hannibal MC, McMillin MJ, Gildersleeve HI, Beck AE, Tabor HK, Cooper GM, Mefford HC, Lee C, Turner EH, Smith JD, Rieder MJ, Yoshiura K, Matsumoto N, Ohta T, Niikawa N, Nickerson DA, Bamshad MJ, Shendure J. Exome sequencing identifies MLL2 mutations as a cause of Kabuki syndrome. Nat Genet 2010; 42: 790-793.

Nussbaum RL, McInnes RR, Willard HF, Hamosh A. (2015). Patterns of single-gene inheritance. En Thompson & Thompson Genetics in Medicine (17, 175). USA: Elsevier.

Oberman AE, Wilson WA, Frasier SD, Donell GN, Bergren WR. Galactokinase-deficiency cataracts in identical twins. Am J Ophthalmol 1972; 74: 887.

Oishi M, Oishi A, Gotoh N, Ogiro K, Higasa K, Iida K, Makiyama Y, Morooka S, Matsuda F, Yoshimura N. Comprensive molecular diagnosis of a large cohort of Japanese retinitis pigmentosa and Usher síndrome patients by next-generation sequencing. Invest Ophthalmol 2014; 55: 7369-75.

Patel N, Hejkal T, Katz A, Margalit E. Ocular manifestations of Donnai-Barrow síndrome. J Child Neurol 2007; 22: 462-4.

Pichi F, Lembo A, Serafino M, Nucci P. Genetics of congenital cataract. Dev Ophthalmol 2016; 57: 1-14.

Penchaszadeh VB, Velazquez D, Arrivillaga R. The nasopalpebral lipoma-coloboma syndrome: a new autosomal dominant displasia-malformation síndrome with congenital nasopalpebral lipomas, eyelid colobomas, telechantus, and maxillary hipoplasia.

Pogue RE, Cavalcanti DP, Shanker S, Andrade RV, Aguiar LR, de Carvalho JL, Costa FF. Rare genetic diseases: update on diagnosis, tratment and online resources. Drug Disc Today 2017 (Epub ahead of print).

Reiners J, Nagel-Wolfrum K, Jürgens K, Märker T, Wolfrum U. Molecular basis of human Usher syndrome: deciphering the meshes of the Usher protein network provides insights into the pathomechanisms of the Usher disease**.** Exp Eye Res. 2006; 83: 97-119.

Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, Grody WW, Hegde M, Lyon E, Spector E, Voelkerding K, Rehm HL. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendations of the American College of Medical Genetics and Genomic and the Association for Meolcular Pathology. Genet Med 2015; 405-424.

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminateing inhibitors. Proc Natl Acad Sci USA 1977; 74: 5463-67.

Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B. (2001). Galactosemia. En The Metabolic & molecular bases of inherited disease (1559-1561). USA: Mc Gran Hill.

Sedano HO, Gorlin RJ. Frontonasal malformation as a field defect and in syndromic associations. Oral Sur Oral Med Oral Path 1988; 65: 704–710.

Sheffield VC, Stone EM. Genomics and eye. N Engl Med 2011; 364: 1932-42.

Shuman C, Beckwith JB, Weksberg R. in: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Mefford HC, Stephens K, Amemiya A, Ledbetter N, editors. Genereviews. Seattle (WA): university of Washington, Seattle; 1993-2017.

Sikkema-Raddatz B, Johansson LF, de Boer EN, et al. Targeted next´generation sequencing can replace Sanger sequencing in clinical diagnostics. Hum Mutat 2013; 34: 1035-42.

Singh G, Lott MT, Wallace DC, Amitichondrial DNA mutation as a cause Leber´s hereditary optic neuropathy. New Engl Med J Med 1989; 320: 1300-5.

Singh R, Ram J, Kaur G, Prasad. Galactokinase deficiency induced cataract in Indian infants: identification of 4 novel mutations in GALK gene. Curr Eye Res 2012; 37: 949-54.

Strachan T, Read A. (2011). Human genetic variability and its consequences. En Human molecular genetics (408-9). USA: Garland Science.

Strande NT, Berg JS. Defining the clinical value of a genomic diagnosis in the era of next-generation sequencing. Annu Rev Genomics Hum Genet 2016; 17: 303-32.

Strömland K, Miller M, Sjögreen L, et al. Oculo-auriculo-vertebral spectrum: associated anomalies, functional déficits and posible developmental risk factors. Am J Med genet A 2007; 143: 1317-25.

Tumer Z, Bach-Holm D. Axenfeld –Rieger syndorme and spectrum of PITX2 and FOXC1 mutations. Eur J Hum Genet 2009; 17: 1527-39.

Van Soest S, Westerveld A, de Jong PT, Bleeker-Wagemakers, EM Berger AA. Retinitis pigmentosa: defined from a molecular point of view. Surv Ophthalmol 1999; 43: 321-34.

Vissers LE, de Ligt J, Gilissen C, Janssen I, Steehouwer M, de Vries P, van Lier B, Arts P, Wieskamp N, del Rosario M, van Bon BW, Hoischen A, de Vries BB, Brunner HG, Veltman JA. A de novo paradigm for mental retardation. Nat Genet 2010; 42: 1109-12.

Wang DY, Chan WM, Tam PO, Baum L, Lam DS, Chong KK, Fan BJ, Pang CP Gene mutations in retinitis pigmentosa and their clinical implications. Clin Chim Acta 2005; 351: 5-16.

Voo I, Small KW. Update on the genetics of macular dystrophies. Retina 2004; 24: 591-601.

Woods CG, Cox J, Springell K, et al. Quantification of homozygosity in consanguineous individuals with autosomal recessive disease. Am J Hum Genet 2006; 78: 889-96.

Xuang J, Yu Y, Qing T, Guo L, Shi L. Next-generation sequencing in the clnic: promises and challenges. Cancer Lett 2013; 340: 284-95.

Yang Y, Muzny DM, Yang Y, Muzny DM, Reid JG, Bainbridge MN, Willis A, Ward PA, Braxton A, Beuten J, Xia F, Niu Z, Hardison M, Person R, Bekheirnia MR, Leduc MS, Kirby A, Pham P, Sucll J, Wang M, Ding Y, Plon SE, Lupski JR, Beaudet AL, Gibss RA, Eng CM. Clinical whole-exome sequencing for the diagnosis of mendelian disorders. N Engl J Med 2013; 369: 1502-11.

Zaghloul NA, Katsanis N. Mechanistic insights into Bardet-Biedl syndrome, a model ciliopathy. J Clin Invest. 2009; 119: 428-3.

RESEARCH LETTER

AMERICAN JOURNAL OF medical genetics PART A

Exome Sequencing Identifies a De Novo Frameshift Mutation in the Imprinted Gene *ZDBF2* in a Sporadic Patient with Nasopalpebral Lipoma-Coloboma Syndrome

Oscar F. Chacón-Camacho,[1] Nara Sobreira,[2] Jing You,[2] Raul E. Piña-Aguilar,[3] Vanessa Villegas-Ruiz,[1] and Juan C. Zenteno[1,4]*

[1]Department of Genetics-Research Unit, Institute of Ophthalmology "Conde de Valenciana", Mexico City, Mexico

[2]Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland

[3]Division of Genomic Medicine, National Medical Center "20 de Noviembre", ISSSTE, Mexico City, Mexico

[4]Faculty of Medicine, Department of Biochemistry, National Autonomous University of Mexico (UNAM), Mexico City, Mexico

Manuscript Received: 19 May 2015; Manuscript Accepted: 6 April 2016

Nasopalpebral lipoma-coloboma syndrome (NPLCS, OMIM% 167730) is an uncommon malformation entity with autosomal dominant inheritance characterized by the combination of nasopalpebral lipoma, colobomas in upper and lower eyelids, telecanthus, and maxillary hypoplasia. To date, no genetic defects have been associated with familial or sporadic NPLCS cases and the etiology of the disease remains unknown. In this work, the results of whole exome sequencing in a sporadic NPLCS patient are presented. Exome sequencing identified a de novo heterozygous frameshift dinucleotide insertion c.6245_6246 insTT (p.His2082fs*67) in *ZDBF2* (*zinc finger, DBF-type containing 2*), a gene located at 2q33.3. This variant was absent in parental DNA, in a set of 300 ethnically matched controls, and in public exome variant databases. This is the first genetic variant identified in a NPLCS patient and evidence supporting the pathogenicity of the identified mutation is discussed.

Key words: nasopalpebral lipoma; coloboma; exome sequencing; *ZDBF2*

How to Cite this Article:
Chacón-Camacho OF, Sobreira N, You J, Piña-Aguilar RE, Villegas-Ruiz V, Zenteno JC. 2016. Exome sequencing identifies a de novo frameshift mutation in the imprinted gene *ZDBF2* in a sporadic patient with Nasopalpebral Lipoma-coloboma syndrome.
Am J Med Genet Part A 170A:1934–1937.

INTRODUCTION

Nasopalpebral lipoma-coloboma syndrome (NPLCS, OMIM% 167730) is a rare malformation syndrome of autosomal dominant inheritance primarily characterized by nasopalpebral lipomas, upper and lower eyelid colobomas, telecanthus, and maxillary hypoplasia. Penchaszadeh et al. [1982] first described the disease in 1982 in a family from Venezuela comprising nine affected subjects in three consecutive generations. To date, two familial [Penchaszadeh et al., 1982; Akarsu and Sayli, 1991] and four sporadic [Bock-Kunz et al., 2000; Moreira Gonzalez and Jackson, 2003; Babu et al., 2011; Chacon-Camacho et al., 2013] NPLCS cases have been reported in the literature. Frequent additional clinical features include broad forehead, displaced, or aplastic lacrimal punctae, persistent epiphora, aberrant eyelashes, conjunctival hyperemia, corneal and lens opacities, and divergent strabismus [Penchaszadeh et al., 1982; Chacon-Camacho et al., 2013]. NPLCS has complete penetrance and a relatively homogeneous phenotype in both familial and sporadic cases.

The pathophysiology of the disorder is unknown and defects in migration of cells of neural crest or a dysplasia of adipose tissue

Present address of Raul E. Piña-Aguilar is Institute of Medical Sciences, University of Aberdeen, Aberdeen, United Kingdom.
Conflict of interest: None.
Grant sponsor: National Human Genome Research Institute; Grant number: 1U54HG006542.
*Correspondence to:
Dr. Juan C. Zenteno, Research Unit, Institute of Ophthalmology, "Conde de Valenciana," Chimalpopoca 14, Col. Obrera, Mexico City, CP 06800, Mexico.
E-mail: jczenteno@institutodeoftalmologia.org
Article first published online in Wiley Online Library (wileyonlinelibrary.com): 3 May 2016
DOI 10.1002/ajmg.a.37683

were previously proposed to explain the aberrant craniofacial development observed in the syndrome [Penchaszadeh et al., 1982]. To date, a specific genetic alteration has not been associated with this Mendelian disorder.

In this work, we report the identification of a de novo truncating mutation in the *ZDBF2* (*zinc finger, DBF-type containing 2*) gene in a patient with the NPLCS detected by whole exome sequencing (WES) and summarize evidence supporting pathogenicity of such mutation.

PATIENT AND METHODS

The propositus is a 2-year-old Mexican girl with a clinical diagnosis of NPLCS (Fig. 1). She was born from unrelated, healthy parents. No family history of congenital malformations or inherited diseases was recorded. The clinical, radiological, and histopathological features of this sporadic case were described by or group [Chacon-Camacho et al., 2013]. In an attempt to identify the genetic defect underlying the syndrome, samples from the proband, and her healthy parents were subjected to WES in the Baylor-Hopkins Centers for Mendelian Genomics (BHCMG).

Briefly, we captured the CCDS exonic regions and flanking intronic regions totaling ~51 Mb by using the Agilent SureSelect Human All Exon V4 51 Mb Kit and performed paired end 100 bp reads on all three family members with the Illumina HiSeq2000 platform. We aligned each read to the reference genome (NCBI human genome assembly build 37; Ensembl core database release 50_36110) with the Burrows-Wheeler Alignment (BWA) tool [Li and Durbin, 2009] and identified single-nucleotide variants (SNVs) and small insertion-deletions (indels) with SAMtools [Li et al., 2009]. We also performed local realignment and base call quality recalibration by using GATK [McKenna et al., 2010; DePristo et al., 2011]. We identified potentially causal variants by standard filtering criteria: SNV and indel minimal depth of 83, root mean square mapping quality of 25, strand bias *P*-value below 104, end distance bias below 104, and filtering out SNVs within 3 bp of an indel and indels within 10 bp of each other; followed by the use of the Analysis Tool of PhenoDB [Sobreira et al., 2015] to design the prioritization strategy.

We prioritized rare functional variants (missense, nonsense, splice site variants, and indels) that were heterozygous de novo mutations, homozygous, or compound heterozygous in the proband and excluded variants with a Minor Allele Frequency (MAF) >0.01 in dbSNP 126, 129, and 131, in the Exome Variant Server (release ESP6500SI-V2), 1000 Genomes Project, or Exome Aggregation Consortium database (ExAC), Cambridge, MA (URL: http://exac.broadinstitute.org). We also excluded all variants found in our in-house controls (CIDRVar 51 Mb). Sanger sequencing confirmed candidate mutations Identified in WES. For distinguishing parental origin of the allele carrying the candidate causal mutation, allele-specific PCR employing primer ASO-RV: 5′-TGGTTGCTCCTATTAAAAAAT G- 3′ (underlined bases indicate a dinucleotide insertion) and Sanger sequencing were used. Complete oligonucleotide sequences and PCR conditions for allele-specific PCR are available on request.

FIG. 1. Facial appearance of the proband at 7 months of age demonstrating the characteristic nasopalpebral lipoma which also involves the frontal region. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmga].

RESULTS

After filtering variants detected by WES, 1 heterozygous de novo variant, 12 compound heterozygous variants in six genes, and one homozygous variant were identified in propositus' DNA (Suppl. Table SI). The de novo variant is a novel heterozygous c.6245_6246insTT (p.His2082fs*67) insertion in the third coding exon of the *ZDBF2* gene (NM_020923), which was absent from parental DNA on WES. Bidirectional Sanger sequencing confirmed that the proband carried the *ZDBF2* dinucleotide insertion (Fig. 2) while her parents exhibited wild-type alleles. The variant has not been reported in the above-mentioned public variant databases and was absent from a set of 300 ethnically matched control alleles genotyped by direct Sanger sequencing. We also identified a second variant in *ZDBF2* in the proband inherited from her mother (c.2086A>C; p.Asn696His) (Suppl. Table I), which was employed for recognition of the maternal *ZDBF2* allele. As shown in supplementary Figure S1, allele-specific sequencing demonstrated that the *ZDBF2* allele carrying the frameshifting dinucleotide insertion was the paternally derived one.

The remaining variants identified in proband's DNA by WES (i.e., a single homozygous mutation and six compound heterozygous mutations) were excluded by its presence in the parents, assuming a dominant mutation model for NPLCS.

DISCUSSION

The pathophysiology of the NPLCS is currently unknown. It has been suggested that the primary defect could involve a dysplasia of adipose tissue leading to nasopalpebral and upper lid lipomas during embryogenesis, with the rest of the malformations being secondary to interference of morphogenesis of the mid-upper face developmental field from the lipomatous hamartomas [Penchas-

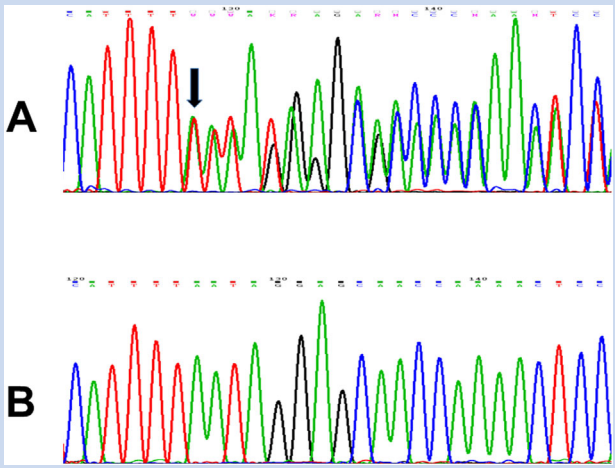

FIG. 2. Partial nucleotide sequence of *ZDBF2* gene. The upper nucleotide sequence (A) corresponds to the proband's DNA demonstrating the heterozygous c.6245_6246insTT insertion (arrow), which predicts a p.His2082fs*67 protein change. Nucleotide sequencing in B is a *ZDBF2* sequence in a control DNA. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmga].

zadeh et al., 1982]. Only two familial cases of the disease have been described and in both pedigrees the phenotype was transmitted as an autosomal dominant trait [Penchaszadeh et al., 1982; Akarsu and Sayli, 1991]. In this work, WES of a sporadic NPLCS case allowed the identification of a de novo truncating mutation in *ZDBF2*, a gene located on human chromosome 2q33.3 and composed of three coding exons that encodes a 2,354 amino acid polypeptide. Both *ZDBF2* mRNA and protein are highly expressed in human tissues as prostate, cerebral cortex, adrenal gland, skin, and adipose tissue, among other body structures (www.proteinatlas.org/ENSG00000204186-ZDBF2/tissue). *ZDBF2* is a nucleic acid binding protein with a DBF-zinc finger domain but its particular function is currently unknown.

Human *ZDBF2* locus was first described to be expressed only from the paternal allele [Kobayashi et al., 2009]. However, recent work has shown that *ZDBF2* is under atypical dynamic imprinting with both maternal and paternal imprinting control during development [Duffié et al., 2014]. *ZDBF2* does not follow the rules of classic imprinted human genes that exhibit acquisition of a differentially methylated region in one of the parental germlines only and have an uninterrupted continuum of paternal or maternal-specific methylation from gametes to adult progeny [Kobayashi et al., 2012; Duffié et al., 2014].

Two lines of evidence support that the heterozygous p.His2082fs*67 truncating *ZDBF2* variant identified in our patient is associated with her malformative phenotype. First, the heterozygous and truncating nature of the *ZDBF2* variant identified in our patient (but not in her healthy parents) is compatible with an autosomal dominant inheritance fitting the transmission pattern described in familial cases of the disease. The variant was absent from a set of 300 ethnically matched control alleles and is not annotated in publicly available databases, including the recently released ExAC, which contains exome data of ~4,000 Mexican people [SIGMA Type 2 Diabetes Consortium, 2014]. Second, loss of *ZDBF2* in humans leads to a similar set of craniofacial malformations that includes some of the anomalies observed in the NPLCS. The DECIPHER database (https://decipher.sanger.ac.uk/) includes seven individuals with 2q33.3 deletions involving *ZDBF2* and six of them feature craniofacial defects including abnormal palpebral fissure and/or eyelashes (IDs 249506, 249239, 249612, 256887, 292523) and prominent/wide nasal bridge (IDs 249506, 256887) (complete data available at https://decipher.sanger.ac.uk/search?q=ZDBF2#consented-patients/results).

It is important to make note that two instances of females transmitting NPLCS to their children have been described in familial cases of the disease [Penchaszadeh et al., 1982; Akarsu and Sayli, 1991], and this fact would invalidate the paternally active-*ZDBF2* being the NPLCS causal gene. However, taking into account the recent findings demonstrating that *ZDBF2* is under atypical dynamic imprinting with both maternal and paternal imprinting control during development [Duffié et al., 2014], the causality of *ZDBF2* in such cases of female transmission cannot be excluded. Alternatively, the possibility of genetic heterogeneity, with mutations in a different gene causing the syndrome, can also be considered in those instances of NPLCS transmission from affected females.

In conclusion, we demonstrated a *ZDBF2* mutation in a NPLCS patient and discussed evidence supporting the pathogenicity of this gene variant. However, genetic analyses of additional patients with this uncommon craniofacial malformation phenotype and/or the development of a knock-out model would provide additional evidence to confirm *ZDBF2* involvement in the NPLCS.

ACKNOWLEDGMENTS

The authors thanks to the Baylor-Hopkins Center for Mendelian Genomics research initiative for whole exome sequencing and data analysis, and the grant from the National Human Genome Research Institute, (1U54HG006542) that provided support for this work.

REFERENCES

Akarsu AN, Sayli BS. 1991. Nasopalpebral lipoma-coloboma syndrome. Clin Genet 40:342–344.

Babu NS, Raviprakash D, Kumar R. 2011. Nasopalpebral lipoma coloboma syndrome. Indian J Ophthalmol 59:379–380.

Bock-Kunz AL, Lyon DB, Singhal VK, Grin TR. 2000. Nasopalpebral lipoma-coloboma. Arch Ophthalmol 118:1699–1701.

Chacon-Camacho OF, Lopez-Martinez MS, Vázquez J, Nava-Castañeda A, Martin-Biasotti F, Piña-Aguilar RE, Iñiguez-Soto M, Acosta-García J, Zenteno JC. 2013. Nasopalpebral lipoma-coloboma syndrome: Clinical, radiological, and histopathological description of a novel sporadic case. Am J Med Genet A 161A:1470–1474.

DePristo MA, Banks E, Poplin R, Garimella KV, Maguire JR, Hartl C, Philippakis AA, del Angel G, Rivas MA, Hanna M, McKenna A, Fennell TJ, Kernytsky AM, Sivachenko AY, Cibulskis K, Gabriel SB, Altshuler D, Daly MJ. 2011. A framework for variation discovery and genotyping using next-generation DNA sequencing data. Nat Genet 43:491–498.

Duffie R, Ajjan S, Greenberg MV, Zamudio N, Escamilla del Arenal M, Iranzo J, Okamoto I, Barbaux S, Fauque P, Bourc'his D. 2014. The Gpr1/ZDBF2 locus provides new paradigms for transient and dynamic genomic imprinting in mammals. Genes Dev 28:463–478.

Kobayashi H, Yamada K, Morita S, Hiura H, Fukuda A, Kagami M, Ogata T, Hata K, Sotomaru Y, Kono T. 2009. Identification of the mouse paternally expressed imprinted gene ZDBF2 on chromosome 1 and its imprinted human homolog ZDBF2 on chromosome 2. Genomics 93:461–472.

Kobayashi H, Sakurai T, Sato S, Nakabayashi K, Hata K, Kono T. 2012. Imprinted DNA methylation reprogramming during early mouse embryogenesis at the Gpr1-ZDBF2 locus is linked to long cis-intergenic transcription. FEBS Lett 586:827–833.

Li H, Durbin R. 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics 25:1754–1760.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, 1000 Genome Project Data Processing Subgroup. 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25:2078–2079.

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA. 2010. The genome analysis toolkit: A mapreduce framework for analyzing next-generation DNA sequencing data. Genome Res 20:1297–1303.

Moreira Gonzalez A, Jackson IT. 2003. Surgical correction of the nasopalpebral lipoma-coloboma syndrome. Eur J Plast Surg 26:263–267.

Penchaszadeh VB, Velasquez D, Arrivillaga R. 1982. The nasopalpebral lipoma-coloboma syndrome: A new autosomal dominant dysplasia malformation syndrome with congenital nasopalpebral lipomas, eyelid colobomas, telecanthus, and maxillary hypoplasia. Am J Med Genet 11:397–410.

SIGMA Type 2 Diabetes Consortium, Estrada K, Aukrust I, Bjørkhaug L, Burtt NP, Mercader JM, García-Ortiz H, Huerta-Chagoya A, Moreno-Macías H, Walford G, Flannick J, Williams AL, Gómez-Vázquez MJ, Fernandez-Lopez JC, Martínez-Hernández A, Centeno-Cruz F, Mendoza-Caamal E, Revilla-Monsalve C, Islas-Andrade S, Córdova EJ, Soberón X, González-Villalpando ME, Henderson E, Wilkens LR, Le Marchand L, Arellano-Campos O, Ordóñez-Sánchez ML, Rodríguez-Torres M, Rodríguez-Guillén R, Riba L, Najmi LA, Jacobs SB, Fennell T, Gabriel S, Fontanillas P, Hanis CL, Lehman DM, Jenkinson CP, Abboud HE, Bell GI, Cortes ML, Boehnke M, González-Villalpando C, Orozco L, Haiman CA, Tusié-Luna T, Aguilar-Salinas CA, Altshuler D, Njølstad PR, Florez JC, MacArthur DG, Jiménez-Morales S. 2014. Association of a low-frequency variant in HNF1A with type 2 diabetes in a Latino population. JAMA 311:2305–2314.

Sobreira N, Schiettecatte F, Boehm C, Valle D, Hamosh A. 2015. New tools for Mendelian disease gene identification: PhenoDB variant analysis module; and genematcher, a web-based tool for linking investigators with an interest in the same gene. Hum Mutat 36:425–431.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Gene 534 (2014) 218–221

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Homozygosity mapping identifies a GALK1 mutation as the cause of autosomal recessive congenital cataracts in 4 adult siblings

Oscar F. Chacon-Camacho [a], Beatriz Buentello-Volante [a], Roberto Velázquez-Montoya [b], Raul Ayala-Ramirez [a], Juan C. Zenteno [a,c,*]

[a] Research Unit-Genetics, Institute of Ophthalmology, "Conde de Valenciana", Mexico City, Mexico
[b] Cornea Department, "Dra. Olga Montoya" Ophthalmic Center, San Jose, Costa Rica
[c] Department of Biochemistry, Faculty of Medicine, National Autonomous University of Mexico, Mexico City, Mexico

ARTICLE INFO

Article history:
Accepted 25 October 2013
Available online 7 November 2013

Keywords:
Homozygosity mapping
Cataract
GALK1
Galactosemia

ABSTRACT

Objective: Monogenic congenital cataract is one of the most genetically heterogeneous ocular conditions with almost 30 different genes involved in its etiology. In adult patients, genotype–phenotype correlations are troubled by eye surgery during infancy and/or long-term ocular complications. Here, we describe the molecular diagnosis of GALK1 deficiency as the cause of autosomal recessive congenital cataract in a family from Costa Rica.
Methods: Four affected siblings were included in the study. All of them underwent eye surgery during the first decade but medical records were not available. Congenital cataract was diagnosed by report. Molecular analysis included genome wide homozygosity mapping using a 250 K SNP Affymetrix microarray followed by PCR amplification and direct nucleotide sequencing of candidate gene.
Results: Genome wide homozygosity mapping revealed a 6 Mb region of homozygosity shared by two affected siblings at 17q25. The *GALK1* gene was included in this interval and direct sequencing of this gene revealed a homozygous c.1144C>T mutation (p.Q382*) in all four affected subjects.
Conclusions: This work demonstrates the utility of homozygosity mapping in the retrospective diagnosis of a family with congenital cataracts in which ocular surgery at early age, the lack of medical records, and the presence of long term eye complications, impeded a clear clinical diagnosis during the initial phases of evaluation.

1. Introduction

Cataract is defined as any opacity of the lens and is the leading cause of irreversible blindness (Foster and Johnson, 1990). There are an estimated 200,000 children blind from cataract worldwide and 20,000 to 40,000 children with developmental bilateral cataract are born each year (Traboulsi, 2012). Defined by age at onset, a congenital or infantile cataract is visible within the first year of life, a juvenile cataract occurs within the first decade of life, a presenile cataract occurs before the age of 45 years, and the senile or age-related cataract occurs thereafter (Francois, 1982). Inherited cataracts usually present early in life and are often assumed to be congenital (Graw, 2004). The genetic background of inherited cataracts is highly heterogeneous and underlying genetic characteristics remain incompletely characterized to date. Cataracts of genetic origin are most often inherited in an autosomal dominant pattern, although autosomal recessive and X-linked inheritance can also occur (Traboulsi, 2012). Inherited cataracts are most often isolated, although they may be associated with other ocular anomalies or be part of multisystem genetic disorders (Haargaard et al., 2004; Hejtmancik, 2008). To date, mutations in 29 genes have been associated with congenital cataracts, 9 of them with autosomal recessive inheritance (Huang and He, 2010; Yasmeen et al., 2010).

Defects in genes controlling basic metabolic pathways may result in cataract formation in association with more complex syndromes such as galactosemia, an autosomal recessive disorder that results from defects in galactokinase (GALK1), galactose-1-phosphate uridyltransferase (GALT), or UDP-galactose-4′-epimerase (GALE), enzymes which convert galactose into glucose-1-phosphate (Foster et al., 1997). The symptoms and severity of the disease are variable and depend on the degree of functional defects of the affected enzyme (Fridovich-Keil and Walter, 2008). GALK1 deficiency (OMIM # 230200) often causes bilateral congenital cataract associated with galactosemia and galactosuria (Segal et al., 1979). The galactosemia-associated cataract is the result of osmotic phenomena caused by the accumulation of galactitol in the lens of newborns exposed to dietary galactose (Asada et al., 1999).

Abbreviations: BRLMM, Bayesian Robust Linear Model with Mahalanobis distance classifier; C, cytosine; DNA, desoxyribonucleic acid; GALK1, galactokinase; GALT, galactose-1-phosphate uridyltransferase; GALE, UDP-galactose-4′-epimerase; Mb, megabase; Ng, nanogram; OMIM, On line Mendelian Inheritance in Man; SNPs, single nucleotide polymorphisms; PCR, polymerase chain reaction; Q, glutamine; T, thymine.

* Corresponding author at: Department of Genetics, Institute of Ophthalmology "Conde de Valenciana", Chimalpopoca 14, Col. Obrera, Mexico City, CP 06800, Mexico. Tel.: +52 55554421700x3212; fax: +52 5555789748.
E-mail address: jczenteno@institutodeoftalmologia.org (J.C. Zenteno).

0378-1119/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2013.10.057

The lack of early remarkable signs of a metabolic disease in galactosemia implies that affected children will not receive medical attention and that their disorder will only be diagnosed after the development of lens opacities or blindness. In most countries, galactosemia is diagnosed as a result of newborn screening. However, in regions where neonatal screening is not available, retrospective diagnosis of GALK1 deficiency in adult patients with early cataract formation is complicated by the development of long term ocular complications such as inflammatory changes, glaucoma, or retinal detachment. In such cases, the application of genomic analysis tools could help to unravel the molecular defect. In this study we describe the successful use of genome wide homozygosity mapping to identify a nonsense mutation in *GALK1* gene as the cause of recessively inherited cataracts in 4 adult patients from a Costa Rican family.

2. Material and methods

2.1. Patients

The family under study came from a small town populated by approximately 1350 inhabitants, in the Alajuela province of Costa Rica. Informed consent for genetic analysis was obtained from all participants and the research was approved by the Institutional Review Board. Genealogical investigation disclosed parental consanguinity (Fig. 1). Four sibs out of 11 (Fig. 1) were reported to suffer from congenital bilateral cataracts during their first decade of life and all of them underwent an ocular surgical procedure at early age (III-3: at 4 years old; III-4: at 11 years old, III-6: at 9 years old, and III-10: at 11 years old). No medical records concerning previous ophthalmologic examinations and procedures were available. Both deceased parents were reported to have normal vision until older ages. At present, all 4 affected subjects are adults in their 60s (III-3 and III-4) and 50s (III-6 and III-10) and were blind (no light perception). Ophthalmologic examination identified bilateral nystagmus (III-3, III-4, and III-6 in Fig. 1), pale optic nerve (III-3 and III-4), retinal detachment (III-6), and glaucoma and corneal edema (III-10). All subjects were surgically aphakic. A systemic examination confirmed that affected individuals did not present associated extra-ocular anomalies or intellectual disability. None of the 4 affected sibs was married nor had descendants.

2.2. Genome-wide linkage analysis

To identify shared regions of homozygosity, a genome-wide linkage scan using Affymetrix 250 K single nucleotide polymorphism (SNP) mapping array (Affymetrix, Inc., Santa Clara, CA) was undertaken in DNA from two affected siblings. Briefly, 250 ng of pooled DNA (125 ng from each patient) was first digested with the *Nsp1* restriction enzyme (New England Biolabs, Boston, MA) and then ligated to adaptors. Each *NspI* adaptor-ligated DNA was amplified in three 100 μl PCR reactions using AmpliTaq Platinum (Clontech Laboratories, Inc., Palo Alto, CA). Fragmented PCR products were then labeled, denatured and hybridized to the array following washing and staining steps on the Affymetrix GeneChip fluidics station 450. Fluorescence intensities were quantified with an Affymetrix array scanner 3000-7G and the data were collected by the Affymetrix GeneChip Operating Software (GCOS) v 1.4. Genotypes were generated using the GTYPE software for BRLMM analysis using default settings. The Homozygosity Mapper software (www.homozygositymapper.org) was used to analyze the genotypes and for the identification of potential region(s) harboring the disease-associated gene (Seelow et al., 2009). Candidate genes within intervals >2 Mb were identified using GeneDistiller software (Seelow et al., 2008), available at www.genedistiller.org.

2.3. GALK1 mutational analysis

Mutations in *GALK1* (Transcript ID *ENSG00000108479*) were screened by direct sequencing using primer pairs for the 8 coding exons of the gene. All exons were amplified by PCR using Hotstart Taq polymerase (Qiagen Mexico, Mexico City, Mexico). Primer sequences and PCR conditions are available on request. PCR products were cleaned up and directly sequenced by means of the Big Dye Terminator Cycle Sequencing System using an ABI PRISM 3130 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

3. Results

3.1. Genetic linkage studies and mutational analysis

Genome-wide SNP data analyzed by Homozygosity Mapper revealed two extended (>2.0 Mb) regions of homozygosity on chromosome 3

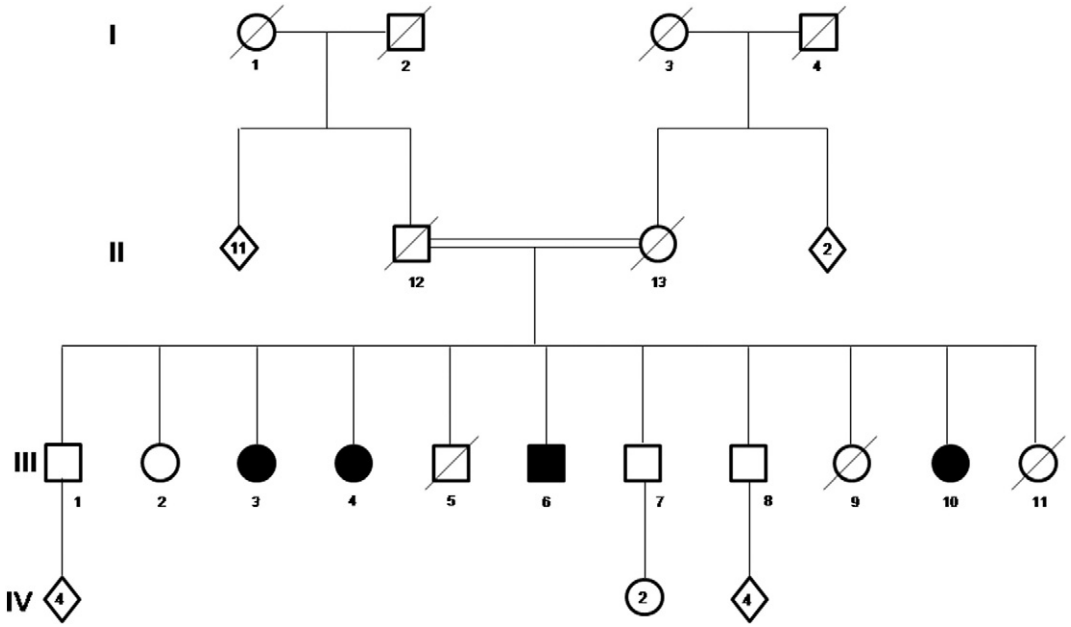

Fig. 1. Genealogy of the congenital cataract family. Solid symbols designate affected subjects. Slash indicates deceased individuals. The squares and circles indicate male and female, respectively; numbers inside diamonds indicate the number of siblings from a specific individual or couple.

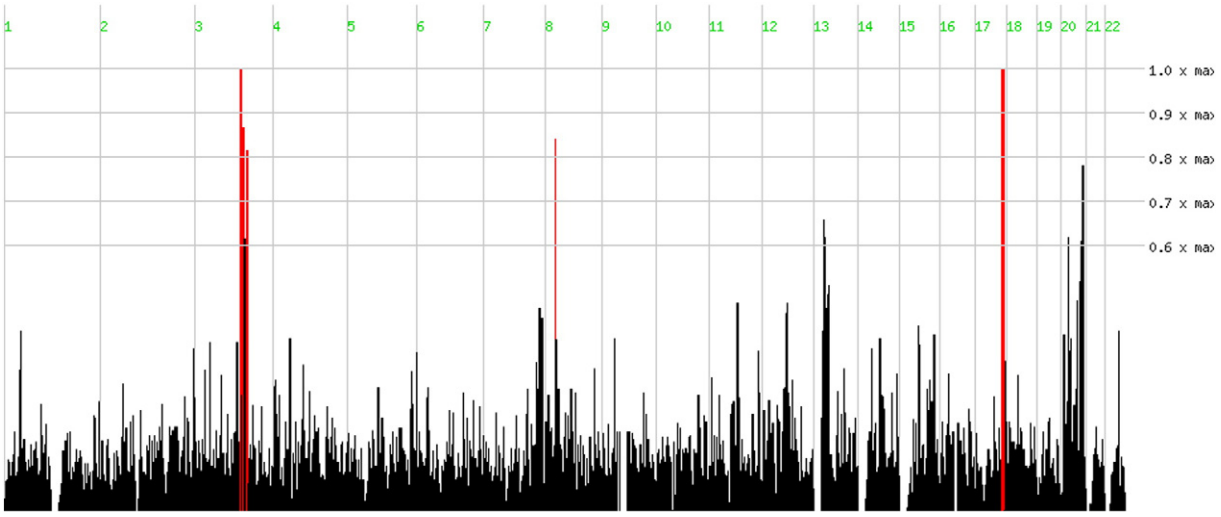

Fig. 2. Genome-wide analysis using Homozygosity Mapper. Genotypes obtained with the Affymetrix 250 K SNP chip were analyzed with the Homozygosity Mapper software for the identification of large stretches of homozygosity. Red top bars indicate homozygous regions identified in pooled DNA from two affected patients. Chromosome numbers (in green color) are shown in the X axis (top). As shown in the screen shot, two chromosomal regions of maximal homozygosity were identified. The largest region corresponded to chromosome 17q25.1 (6.6 Mb; from rs903101 to rs4313838) and contained 163 known genes, pseudogenes, and hypothetical proteins genes. The *GALK1* gene, associated with congenital and juvenile cataract, was located within this candidate interval.

(2.76 Mb; from rs117407287 to rs120165394), and chromosome 17 (6.6 Mb; from rs903101 to rs4313838), shared by both affected siblings (Fig. 2). No ocular disease genes were identified within the 2.76 Mb region on chromosome 3. The 6.6 Mb chromosomal region at 17q25.1 contained 163 known genes, pseudogenes, and hypothetical proteins genes. The *GALK1* gene, associated with congenital and juvenile cataract, was located within this candidate interval. Direct sequencing of *GALK1* was carried out in DNA from one affected subject and a homozygous c.1144C>T point mutation was detected in exon 8 of this gene. This mutation predicts a nonsense change at amino acid residue 382 (p.Q382*) of the GALK protein (Fig. 3). The p.Q382* mutation was found to be homozygous in all 4 affected individuals. DNA analysis in the four unaffected siblings revealed that they were homozygous for the wild type GALK1 allele.

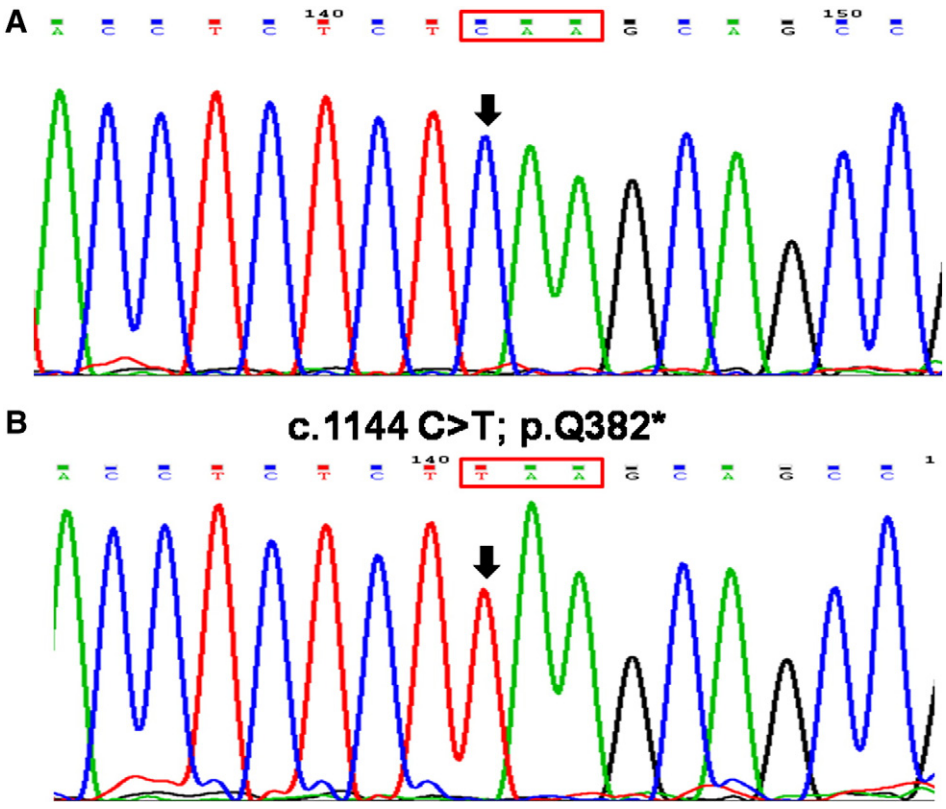

Fig. 3. Partial nucleotide sequence of *GALK1* gene exon 8 in DNAs from a control subject (A) and from one of the congenital cataract siblings (B). A homozygous c.1144C>T mutation is observed in patient's DNA (arrowed nucleotide). This substitution predicts a p.Q382* nonsense mutation at the protein level.

4. Discussion

Inherited congenital cataract is one of the principal causes of treatable impaired vision in pediatric patients and one of the most genetically heterogeneous ocular disorders. To date, autosomal dominant, recessive, or X-linked cataractogenic mutations have been identified in approximately 29 distinct genes (Huang and He, 2010; Yasmeen et al., 2010).

In this study, we localized the candidate locus of an autosomal recessive Costa Rican congenital cataract pedigree using genome wide homozygosity mapping and identified a homozygous p.Q382* nonsense mutation in the GALK1 gene as the cause of the disease. As all four affected sibs in this family underwent ocular surgery during childhood and their medical records were not available, a phenotype-driven molecular investigation was complicated. Instead, a retrospective diagnosis of GALK deficiency was made using a homozygosity mapping (autozygosity) strategy followed by sequencing of candidate gene within the linked region. Our work offers an additional example of the value of homozygosity mapping in the discovery of the genetic basis of recessive disorders (Alkuraya, 2010, 2012; Littink et al., 2012).

Galactosemia is an autosomal recessive disorder caused by a deficiency of either galactokinase, galactose-1-phosphate uridyltransferase, or uridine diphosphate galactose 4-epimerase. In contrast to classical galactosemia caused by transferase deficiency, patients with galactokinase deficiency exhibit early onset cataracts as the major clinical manifestation (Fridovich-Keil and Walter, 2008).

The p.Q382* nonsense mutation identified in the affected siblings predicts a premature termination and the resulting protein lacks its C-terminal amino acids, which are critical for galactokinase activity (Kolosha et al., 2000). The Q382* mutation identified in this pedigree is identical to that previously reported by Kolosha et al. (2000) in 6 apparently unrelated GALK deficient children from Costa Rica identified by neonatal screening. Although it was not possible to relate those patients with the affected subjects described in our pedigree, these results suggest a founder mutation effect for the cataractogenic *GALK1* Q382* mutation in Costa Rica.

Mutations in *GALK1* as the cause of familial cataracts were first demonstrated in 1995 by Stambolian et al. (1995) and since then, approximately 35 *GALK1* mutations, most of them missense changes, have been demonstrated in patients with congenital cataracts from different countries (mutation list available www.hgmd.cf.ac.uk). Recently, a p.L139P mutation was identified in a large family from Pakistan segregating autosomal recessive congenital cataracts using a genome-wide scan with polymorphic microsatellite markers (Yasmeen et al., 2010).

Congenital cataracts are genetically and phenotypically heterogeneous. The relationships between genotype and phenotype are complex and a specific clinical cataract phenotype may be the result of mutations in several different genes. Although more than 20 genes have been identified to underlie monogenic forms of non-syndromic cataracts, there is possibly even more heterogeneity with more genes remaining to be identified and thus the clinical and molecular analysis of additional families from distinct ethnic origins is warranted.

In conclusion, this work demonstrates the utility of homozygosity mapping in the retrospective diagnosis of a family with congenital cataracts in which ocular surgery at early age, the lack of medical records, and the presence of long term eye complications, situations which are still common in several countries, impeded a clear clinical diagnosis during the initial phases of evaluation.

Conflict of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgments

The authors thank Ferdinand Hendlmeier for critical review of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2013.10.057.

References

Alkuraya, F.S., 2010. Homozygosity mapping: one more tool in the clinical geneticist's toolbox. Genet. Med. 12, 236–239.
Alkuraya, F.S., 2012. Discovery of rare homozygous mutations from studies of consanguineous pedigrees. Curr. Protoc. Hum. Genet. 75, 6.12.1–6.12.13.
Asada, M., Imamura, T., Suyama, I., Hase, Y., Isshiki, G., 1999. Molecular characterization of galactokinase deficiency in Japanese patients. J. Hum. Genet. 44, 377–382.
Foster, A., Johnson, G.J., 1990. Magnitude and causes of blindness in the developing world. Int. Ophthalmol. 14, 135–140.
Foster, A., Gilbert, C., Rahi, J., 1997. Epidemiology of cataract in childhood: a global perspective. J. Cataract Refract. Surg. 23, 601–604.
Francois, J., 1982. Genetics of cataract. Ophthalmologica 184, 61–71.
Fridovich-Keil, J.L., Walter, J.H., 2008. Galactosemia. In: Scriver, Childs, Sly, Valle, Beaudet, Vogelstein, Kinzier, Antonarakis, Ballabio (Eds.), The On Line Metabolic and Molecular Bases of Inherited Disease, 72, pp. 1–92 (Accessed August 2013.Availablefrom: http://www.ommbid.com/OMMBID/the_online_metabolic_and_molecular_bases_of_inherited_disease/b/abstract/part7/ch72).
Graw, J., 2004. Congenital hereditary cataracts. Int. J. Dev. Biol. 48, 1031–1044.
Haargaard, B., Wohlfahrt, J., Fledelius, H.C., Rosenberg, T., Melbye, M., 2004. A nationwide Danish study of 1027 cases of congenital/infantile cataracts: etiological and clinical classification. Ophthalmology 111, 2292–2298.
Hejtmancik, J.F., 2008. Congenital cataracts and their molecular genetics. Semin. Cell Dev. Biol. 19, 134–149.
Huang, B., He, W., 2010. Molecular characteristics of inherited congenital cataracts. Eur. J. Med. Genet. 53, 347–357.
Kolosha, V., et al., 2000. Novel mutations in 13 probands with galactokinase deficiency. Hum. Mutat. 15, 447–453.
Littink, K.W., den Hollander, A.I., Cremers, F.P., Collin, R.W., 2012. The power of homozygosity mapping: discovery of new genetic defects in patients with retinal dystrophy. Adv. Exp. Med. Biol. 723, 345–351.
Seelow, D., Schwarz, J.M., Schuelke, M., 2008. Genedistiller—distilling candidate genes from linkage intervals. PLoS One 3, e3874.
Seelow, D., Schuelke, M., Hildebrandt, F., Nürnberg, P., 2009. Homozygosity Mapper—an interactive approach to homozygosity mapping. Nucleic Acids Res. 37, W593–W599.
Segal, D., Rutman, J.Y., Frimpter, G.W., 1979. Galactokinase deficiency and mental retardation. J. Pediatr. 95, 750–752.
Stambolian, D., et al., 1995. Cloning of the galactokinase cDNA and identification of mutations in two families with cataracts. Nat. Genet. 10, 307–312.
Traboulsi, E.I., 2012. Genetic Disease of the Eye. Oxford University Press, New York.
Yasmeen, A., et al., 2010. Autosomal recessive congenital cataract in consanguineous Pakistani families is associated with mutations in GALK1. Mol. Vis. 16, 682–688.

Gene 528 (2013) 178–182

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Exome sequencing identifies *RDH12* compound heterozygous mutations in a family with severe retinitis pigmentosa

Oscar F. Chacon-Camacho [a], Serguei Jitskii [b], Beatriz Buentello-Volante [a], Jonathan Quevedo-Martinez [a], Juan C. Zenteno [a,c,*]

[a] Department of Genetics-Research Unit, Institute of Ophthalmology "Conde de Valenciana", Mexico City, Mexico
[b] Retinal Pathology Treatment Center "Mexico with Vision" Fund, Nezahualcoyotl, México
[c] Department of Biochemistry, Faculty of Medicine, UNAM, Mexico City, Mexico

ARTICLE INFO

Article history:
Accepted 2 July 2013
Available online 27 July 2013

Keywords:
Retinitis pigmentosa
Exome sequencing
RDH12
Retinal dystrophy
Compound heterozygosity

ABSTRACT

Objective: Retinitis pigmentosa (RP) is the most prevalent type of inherited retinal degeneration and one of the commonest causes of genetically determined visual dysfunction worldwide. To date, approximately 35 genes have been associated with nonsyndromic autosomal recessive RP (arRP), however the small contribution of each gene to the total prevalence of arRP and the lack of a clear genotype–phenotype correlation complicate the genetic analysis in affected patients. Next generation sequencing technologies are powerful and cost-effective methods for detecting causative mutations in both sporadic and familial RP cases.
Methods: A Mexican family with 5 members affected from arRP was studied. All patients underwent a complete ophthalmologic examination. Molecular methods included genome-wide SNP homozygosity mapping, exome sequencing analysis, and Sanger-sequencing confirmation of causal mutations.
Results: No regions of shared homozygosity among affected subjects were identified. Exome sequencing in a single patient allowed the detection of two missense mutations in the *RDH12* gene: a c.446T>C transition predicting a novel p.L149P substitution, and a c.295C>A transversion predicting a previously reported p.L99I replacement. Sanger sequencing confirmed that all affected subjects carried both *RDH12* mutations.
Conclusions: This study adds to the molecular spectrum of *RDH12*-related retinopathy and offers an additional example of the power of exome sequencing in the diagnosis of recessively inherited retinal degenerations.

1. Introduction

Retinitis pigmentosa (RP) is an inherited retinal disorder in which progressive loss of photoreceptors, predominantly rods, leads to night blindness, peripheral visual field loss, and, at later stages, to irreversible blindness. Fundus examination findings in affected individuals include bone-spicule pigmentation, retinal blood vessel attenuation, and waxy pallor of the optic disk (Berson, 1993). The disease has an estimated prevalence of 1 in 3500–4000 (Bunker et al., 1984) and is a prototypic example of a genetically heterogeneous disorder as autosomal dominant, autosomal recessive, X-linked, or mitochondrial modes of inheritance can be observed in affected families (Hartong et al., 2006). To date, about 60 genes involved in human non-syndromic RP have been recognized (RetNet, at https://sph.uth.edu/retnet).

The most common and genetically heterogeneous RP subtype is the autosomal recessive form (arRP), with more than 35 genes implicated. Nonetheless, this group of identified genes is estimated to account for only 30 to 60% of cases with this mode of inheritance (Daiger et al., 2007; den Hollander et al., 2010). Due to this extraordinary locus heterogeneity, complete sequence analysis of all known arRP genes is time consuming and expensive and therefore, several approaches have been developed to uncover the causal mutation in familial and sporadic cases of the disease. For example, about one third of arRP genes were discovered by homozygosity mapping combined with a candidate gene selection approach (Bandah-Rozenfeld et al., 2010; Collin et al., 2011).

In arRP pedigrees that are not suitable for linkage mapping or in sporadic cases of unknown mode of transmission, next generation sequencing methodologies offer efficient strategies to identify the genetic basis of the disease (Tucker et al., 2011; Wang et al., 2012; Zuchner et al., 2011).

Abbreviations: A, adenine; arRP, autosomal recessive retinitis pigmentosa; Bp, base pairs; BRLMM, Bayesian robust linear model with Mahalanobis distance classifier; C, cytosine; DNA, desoxirribonucleic acid; ERG, electroretinogram; FAG, fluorescein retinal angiography; GCOS, GeneChip Operating Software; I, isoleucine; L, leucine; LCA, Leber congenital amaurosis; LM-PCR, ligation mediated-PCR; Logmar, logarithm of the minimum angle of resolution; MgCl2, magnesium chloride; NAD(P), nicotinamide adenine dinucleotide phosphate; Ng, nanogram; OCT, optical coherence tomography; P, proline; PCR, polymerase chain reaction; PolyPhen2, polymorphism phenotyping 2; RDH12, retinol dehydrogenase 12; RP, retinitis pigmentosa; SIFT, sorting intolerant from tolerant; SNP, single nucleotide polymorphism; T, thymine; μl, microliter.

* Corresponding author at: Genetics, Institute of Ophthalmology "Conde de Valenciana", Chimalpopoca 14, Col. Obrera, Mexico City 06700, Mexico. Tel./fax: +52 55 54 42 17 00x3212.
E-mail address: jczenteno@institutodeoftalmologia.org (J.C. Zenteno).

0378-1119/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.gene.2013.07.021

Here, we employed whole-exome sequencing to uncover the genetic cause of arRP in an inbred Mexican family in which homozygosity mapping failed to identify the disease locus. Compound heterozygosity for *RDH12* gene mutations was recognized as the origin of the disease. Our results show that exome sequencing is a powerful approach for molecular diagnostics of arRP families where autozygosity fails to identify the disease locus due to allelic heterogeneity.

2. Materials and methods

2.1. Subjects

The study was approved by the Institutional Review Board of the Institute of Ophthalmology "Conde de Valenciana", at Mexico City. All patient samples were collected with written informed consent and clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki. A three generation family was studied and although the parents of affected subjects in the third generation were not aware of a common ancestry, they originate from the same small isolated village (currently ~8000 inhabitants), making a distant relationship possible. In addition, the absence of cases in previous generations, the consanguinity (first cousins) of the asymptomatic parents of an affected subject in the fourth generation IV-7 (Fig. 1), and the fact that males and females were diseased, strongly suggested autosomal recessive inheritance of RP.

Ophthalmologic examination of four affected siblings (III-1, III-2, III-3, and III-6, Fig. 1) and their parents (II-5 and II-6) and of the one affected nephew in the fourth generation (IV-7) included best-corrected visual acuity, slit-lamp biomicroscopy, fundus examination and photography, fundus fluorescein angiography, optical coherence tomography (OCT) for assessment of retinal thickness, and full-field flash electroretinograms (ERGs). ERGs were recorded following the standards of the International Society for Clinical Electrophysiology of Vision. Scotopic rod-driven responses and cone-driven photopic single flash and 30 Hz flicker stimuli were recorded sequentially.

2.2. Whole-genome SNP homozygosity mapping

A genome-wide linkage scan using an Affymetrix 250K NspI single nucleotide polymorphism (SNP) mapping array (Affymetrix, Inc., Santa Clara, CA) was undertaken to identify shared regions of homozygosity, as previously described (Zenteno et al., 2011). Briefly, 250 ng of pooled genomic DNA (83 ng from each of patients III-3, III-6, and IV-7, in Fig. 1) were first digested with the NspI restriction enzyme (New England Biolabs, Boston, MA) and then ligated to adaptors. Each NspI adaptor-ligated DNA was amplified in three 100 μl PCR reactions using AmpliTaq Platinum (Clontech Laboratories, Inc., Palo Alto, CA). Fragmented PCR products were then labeled, denatured and hybridized to the array following washing and staining steps on the Affymetrix GeneChip fluidics station 450. Fluorescence intensities were quantified with an Affymetrix array scanner 3000-7G and the data were collected by the Affymetrix GeneChip Operating Software (GCOS) v 1.4. Genotypes were generated using the GTYPE software for BRLMM analysis using default settings. The HomozygosityMapper software (www.homozygositymapper.org) was used to analyze the genotypes and for the identification of potential region(s) harboring the disease-associated gene (Seelow et al., 2009). Candidate regions within intervals >2 Mb were identified using GeneDistiller software (Seelow et al., 2008), available at www.genedistiller.org.

2.3. Exome capture and sequencing

Exome sequencing was performed on a single RP patient from this family (IV-7) by Ambry Genetics, Aliso Viejo, CA, USA. Samples were prepared using Illumina's protocol TruSeq DNA Sample Preparation Guide. Briefly, samples were sheared to an average size of 300–400 bp using sonication. DNA fragment ends were repaired and phosphorylated using Klenow, T4 DNA Polymerase and T4 Polynucleotide Kinase. Next, an 'A' base was added to the 3′ end of the blunted fragments, followed by ligation of Illumina Paired-End adapters via T-A mediated ligation. From here, samples were prepared using the NimbleGen protocol outlined in "NimbleGen SeqCap EZ Exome Library SR User's Guide" (Version 3.0). The libraries were amplified using LM-PCR and 1ug of amplified sample libraries were hybridized with Nimblegen's Exome Library baits for 64 h at 47 °C. Captured DNA was then washed and recovered using Streptavidin Dynabeads. The captured DNA was LM-PCR amplified for a total of 17 cycles. The amplified capture DNA library size and concentration were determined using an Agilent bioanalyzer. The captured library was then loaded on a HiSeq 2000 platform for sequencing with a mean exome coverage of 30×. Raw image files were processed by Illumina Pipeline v1.7 for base calling. SNPs and indels were called using an in-house developed software (Ambry Genetics). Identified variants were filtered against the Single Nucleotide Polymorphism database (dbSNP 129, http://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi), 1000 genomes project (www.1000genomes.org), and Exome Variant Server (http://evs.gs.washington.edu/EVS/) databases.

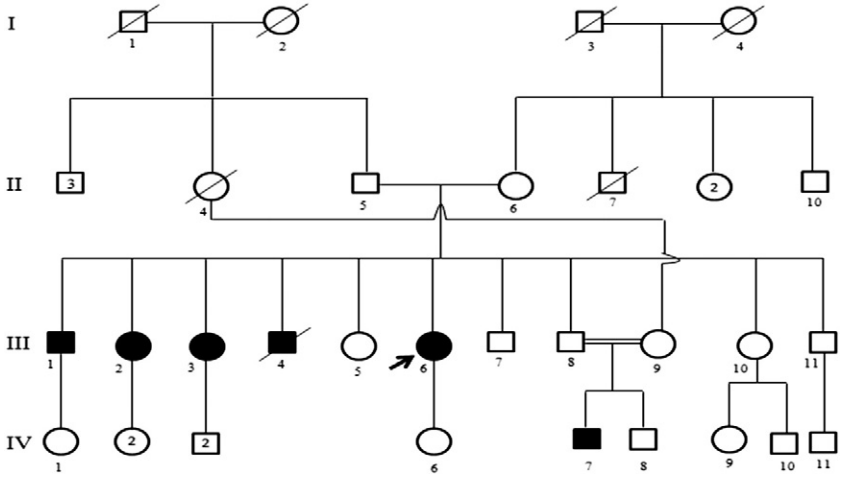

Fig. 1. Genealogy of the RP family showing the segregation of the p.L99I and p.L149P RDH12 mutations. Solid symbols designate affected subjects. Arrow indicates the propositus and slash indicates deceased individuals. Note that III-8 and III-9 are first cousins.

2.4. Analysis of novel exomic variants

Novel missense variants were evaluated in silico using the PolyPhen2 (Polymorphism Phenotyping, available at http://genetics.bwh.harvard.edu/pph2/) and SIFT (Sorting Intolerant from Tolerant, available at http://sift.jcvi.org/) programs to analyze sequence conservation, chemical change, and likelihood of pathogenicity, to predict their possible impact on the structure and function of the protein. Detected variations were also investigated in the 1000 Genomes and Exome Variant Server databases for excluding them as common polymorphisms.

2.5. PCR and Sanger sequencing for familial segregation analysis of RDH12 variants

Genomic DNA was extracted from peripheral blood leukocytes using a semiautomated Quickgene system (Fujifilm, Tokyo, Japan). The exons 3 and 4 of *RDH12* were amplified by PCR using pairs of primers derived from gene normal sequences (exon 3 Fwd: 5′-ATG GCT GGG AGA ATG AAT GC-3′; Rev: 5′-CAT AGA TGG TCT AGG GTG GA-3′; and exon 4 Fwd: 5′-TAT GCA GGT CTG TTA CAG GC-3′, Rev: 5′-CCC TGG ACA TTC TCC ACA TT-3′, *Ensembl ID 00000539142*). Each 25 μl PCR amplification reaction contained 1× buffer, 100 ng of genomic DNA, 0.2 mM of each dNTP, 2 U Taq polymerase, 1 mM of forward and reverse primers, and 1.5 mM MgCl2. PCR products were analyzed in 1.5% agarose gels from which the bands with the amplified templates were excised and the DNA was subsequently purified with the help of Qiaex II kit (Qiagen, Hilden, Germany). Direct automated sequencing of both exons of *RDH12* was performed with the BigDye Terminator Cycle Sequencing kit (Apllied Biosystems, Foster City, CA, USA). All samples were analyzed on an ABI 3130 Genetic Analyzer (Applied Biosystems). Wild-type and mutated *RDH12* sequences were compared manually. Familial segregation of both variants was analyzed.

3. Results

3.1. Clinical assessment

Ophthalmic examination identified four siblings (III-1, III-2, III-3, and III-6) and a nephew (IV-7) affected from arRP (Fig. 1), among 12 examined family members. Affected subjects in this family exhibited similar clinical features. All patients had a progressive bilateral decrease of visual acuity and photophobia since the first decade of the life. Actual mean visual is 0.04 LogMAR (range: 0.0005–0.06). Fundus and FAG examination showed pale optic discs, narrowing of the arterioles, dense intraretinal pigment migration, severe macular retinal pigment epithelium atrophy, severe atrophic pigmentary maculopathy and spicular pigmentations in periphery (Fig. 2A–F). OCT scan demonstrated retinal atrophy with marked macular thinning. Severe cystoid macular edema was also demonstrated in patient III-6 (Fig. 2G). ERG showed undetectable or severely attenuated rod and cone responses (Fig. 2H), indicating severe generalized retinal dysfunction from a young age.

3.2. Genome-wide SNPs array Homozygosity mapping analysis

The initial genotyping did not identify any regions of homozygosity containing any known arRP gene. A single region of ~3 Mb was identified at chromosome 18p11.2 but it does not contain any obvious RP candidate gene after analysis with the HomozygosityMapper and GeneDistiller software. The *TUBB6* gene, encoding a beta-tubulin isotype, was initially chosen as a candidate gene within this region but no mutations were identified after sequencing it in DNA from an affected subject (data not shown).

3.3. Exome sequencing results

Exome sequencing identified 88,831 SNPs and 1702 indels in DNA from the single arRP affected subject analyzed (IV-7, Fig. 1), which were then filtered against several public variation databases. Homozygous mutations were identified in 69 different genes but none of them was related to retinal disease nor were apparently involved in retinal function. This led us to investigate the possibility of recessive compound heterozygous inheritance. Compound heterozygous mutations were identified in 33 genes, including *RDH12*, which was the only gene within this group that is associated with arRP (Janecke et al., 2004). The first *RDH12* mutation was a c.295C>A transversion at exon 3, predicting a previously reported p.L99I substitution (Thompson et al., 2005); the second mutation was a c.446T>C transversion at exon 4 which predicts a novel p.L149P substitution. In addition, exome analysis did not reveal any potential pathogenic mutation in the 18p11 chromosome 3 Mb region initially identified by means of homozygosity mapping, confirming that this locus is not associated to the disorder in the pedigree.

3.4. Novel RDH12 variant analysis

The novel p.L149P RDH12 mutation was predicted to be damaging by both SIFT and Polyphen programs with scores of 0 and 1, respectively

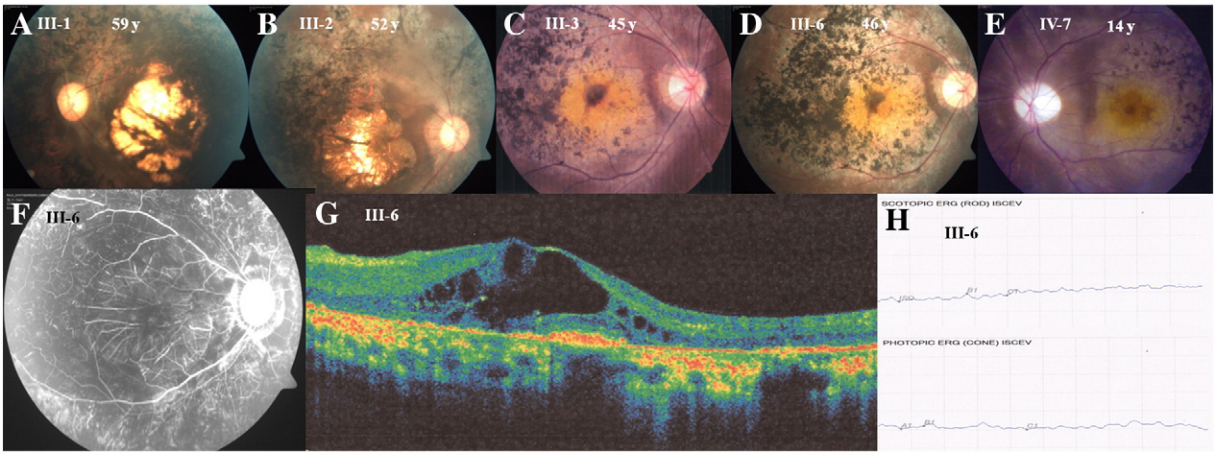

Fig. 2. Ocular phenotype in affected patients. (A–E) Funduscopic features include dense intraretinal pigment migration, severe retinal pigment epithelium atrophy, and arteriolar attenuation, with a severe atrophic pigmentary maculopathy. Note that the degree of macular atrophy is age-dependent. (F) Fluorescein angiography images evidencing retinal vascular attenuation and chorioretinal atrophy. (G) OCT demonstrates retinal atrophy and severe cystoid macular edema. (H) ERG shows no detectable rod or cone responses.

(protein ID ENSP00000449079), and was not annotated at *1000 genomes* or *Exome Variant Server* databases.

3.5. Verification of familial segregation of RDH12 variants

All available family members (II-5, II-6, III-1, III-2, III-3, III-5, III-6, III-8, III-9 and IV-7) were then screened by PCR amplification and Sanger sequencing for the two *RDH12* mutations, c.295C>A and c.446T>C (Fig. 3). Genetic analysis demonstrated that only arRP affected patients carried both mutations (Fig. 4).

4. Discussion

RP is one of the commonest causes of genetically determined visual dysfunction worldwide. To date, mutations in over 60 genes have been implicated in RP, and this makes it one of the most genetically heterogeneous families of disease (Ferrari et al., 2011). The identification of causative genes for RP has been challenging due to the significant overlap in disease phenotypes and the lack of sufficient information on phenotype–genotype associations. Therefore, there has been partial success with approaches of screening of known RP candidate genes by conventional Sanger sequencing. Recent advances in exon capture and massively parallel high-throughput DNA sequencing enabled scanning of the protein-coding regions, the exome, of virtually all genes which comprise ≈1% to 2% of the genome (Ng et al., 2010). Exome sequencing has come to the aid by enabling the identification of RP-associated mutations by sequencing the whole exome of a small number of affected in a family or even in sporadic cases (Khateb et al., 2012; Tucker et al., 2011; Wang et al., 2012; Zuchner et al., 2011).

Currently, the most useful approach for the identification of the disease locus in arRP is homozygosity mapping using genome-wide SNPs genotyping (Bandah-Rozenfeld et al., 2010). This technique has proven to be more effective in inbred families as consanguinity is known to render a percentage of the genome homozygous by descent that is directly correlated to the degree of consanguinity. However, allelic heterogeneity (i.e., different mutations occurring within a single gene) can occur even in consanguineous families, impeding locus identification by autozygosity. Allelic heterogeneity is a known pitfall for homozygosity mapping of retinal dystrophies and has been recognized in a number of previous studies (Banayoun et al., 2009; Ducroq et al., 2006; Miano et al., 2000). Given the low levels of homozygosity obtained after SNP array homozygosity mapping in this family, the possibility of compound heterozygosity was considered and we decided to perform exome sequence analysis in a single affected individual from this pedigree. Compound heterozygous mutations in the *RDH12* gene, at chromosome 14q23, were demonstrated to be the cause of the retinal phenotype in this inbred family. A previously known p.L991 substitution and a novel p.L149P missense mutation in RDH12 were identified in compound heterozygous state in all five affected subjects in this family. Both substitutions are located at the NAD(P)-binding domain of RDH12 protein, which is involved in nucleotide binding.

Mutations in *RDH12* as a cause of retinal dystrophy were first described by Janecke et al. (2004) and by Perrault et al. (2004) in patients with Leber congenital amaurosis (LCA) or early-onset retinal dystrophy, respectively. *RDH12* encodes a member of the superfamily of short-chain alcohol dehydrogenases/reductases and is expressed predominantly in the inner segments of photoreceptors (Haeseleer et al., 2002) where it plays a critical role in the interconversion of vitamin A (all-*trans* retinal) to 11-*cis* retinal, the light-absorbing chromophore of rhodopsin and cone opsins. To date, approximately 65 different *RDH12* mutations, most of them missense, have been demonstrated predominantly in LCA patients (Perrault et al., 2004; Sun et al., 2007; Valverde et al., 2009) but also in early-onset retinal dystrophy (Janecke et al., 2004) and in one family with autosomal-dominant RP (Fingert et al., 2008). It is estimated that *RDH12* mutations account for approximately 3–7% of cases of autosomal recessive retinal dystrophy (Mackay et al., 2011; Thompson et al., 2005; Valverde et al., 2009).

The fundus phenotype in patients carrying *RDH12* mutations is characterized by widespread retinal pigment epithelium (RPE) atrophy with minimal intraretinal pigment migration, and maculopathy at younger ages and striking intraretinal pigmentation, severe pigmentary maculopathy, yellow macular deposits, and macular excavation, as the patients age (27). These retinal features coincided with those observed in both the young and adult patients in our study. In addition, severe cystic macular edema was shown by OCT in adult patient III-6, a feature also previously described in *RDH12*-associated retinal disease (24). Based on the present and previous reports, it appears that either homozygous or compound heterozygous RDH12 mutations cause a very similar fundus phenotype (MacKay et al., 2011; Schuster et al., 2007). Although it can be argued that the retinal phenotype could guide us to initially choose *RDH12* as a candidate gene in this family, it is also well known that a strict genotype–phenotype correlation does not occur in

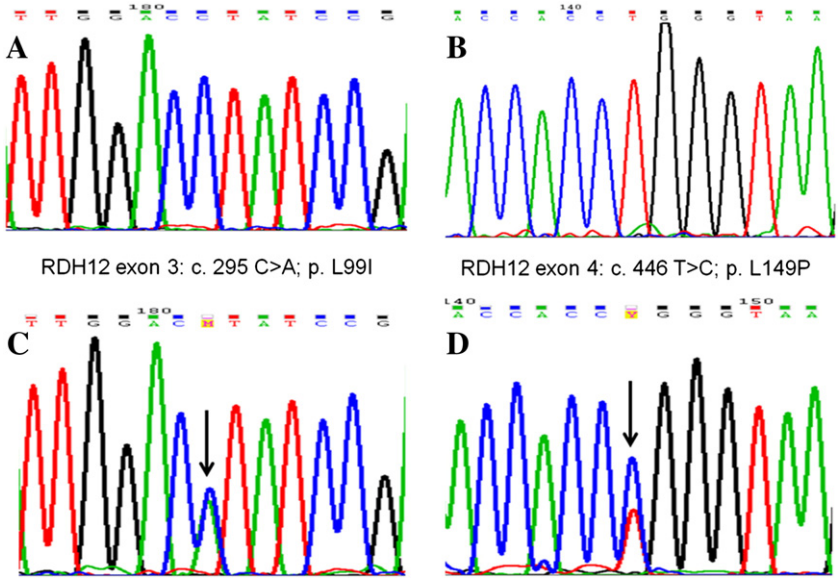

Fig. 3. *RDH12* mutational analysis by Sanger sequencing. (C) A heterozygous c.295C>A transversion (arrow) was demonstrated in *RDH12* exon 3 in DNA from an affected RP patient. (D) A novel heterozygous c.446T>C transition (arrow) was identified in exon 4 of the other *RDH12* allele. Normal *RDH12* sequences are shown for comparison in A and B.

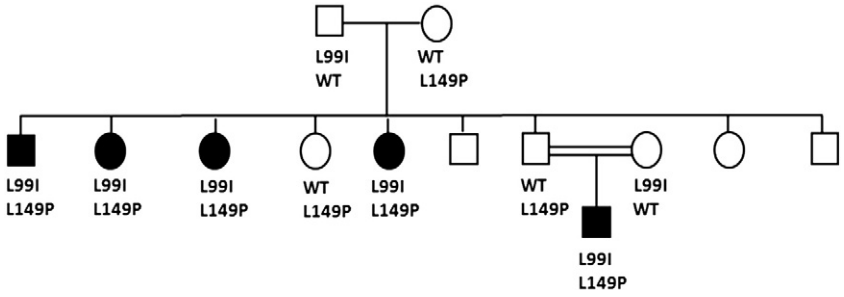

Fig. 4. Simplified pedigree of the arRP family showing the segregation of the p.L99I and p.L149P RDH12 mutations. Filled symbols represent affected patients; WT indicates wild type sequence.

RP. Moreover, linkage analysis was negative for 14q, the RDH12 locus, suggesting the involvement of this gene unlikely.

The a priori assumption is that when a recessive disease arises in a consanguineous family, it must be due to a mutation that is identical-by-descent. Nevertheless, while the relative frequency of an AR disease in an isolated population (or in an extended family, as the one described here) usually suggests a founder effect, in certain cases the high frequency is due to more than one mutation in either one or several genes (Zlotogora, 2007). Compound heterozygosity in a consanguineous family can be attributed to the increased force of genetic drift in inbred populations (Carrasquillo et al., 1997).

Our study shows that exome analysis in a single affected subject from this family efficiently identified causative compound heterozygous mutations in *RDH12*. This work offers an additional example of the power of exome sequencing for the identification of causative mutations in arRP pedigrees. It can be anticipated that in the next few years, this technology will allow the identification of numerous cases of arRP caused by compound heterozygosity (either in non-consanguineous or consanguineous families), which is not amenable of mapping by homozygosity or by other linkage-based positional cloning methods.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

References

Bandah-Rozenfeld, D., et al., 2010. Homozygosity mapping reveals null mutations in FAM161A as a cause of autosomal-recessive retinitis pigmentosa. Am. J. Hum. Genet. 87, 382–391.

Benayoun, L., et al., 2009. Genetic heterogeneity in two consanguineous families segregating early onset retinal degeneration: the pitfalls of homozygosity mapping. Am. J. Med. Genet. A 149A, 650–656.

Berson, E.L., 1993. Retinitis pigmentosa. The Friedenwald Lecture. Invest. Ophthalmol. Vis. Sci. 34, 1659–1676.

Bunker, C.H., Berson, E.L., Bromley, W.C., Hayes, R.P., Roderick, T.H., 1984. Prevalence of retinitis pigmentosa in Maine. Am. J. Ophthalmol. 97, 357–365.

Carrasquillo, M.M., Zlotogora, J., Barges, S., Chakravarti, A., 1997. Two different connexin 26 mutations in an inbred kindred segregating non-syndromic recessive deafness: implications for genetic studies in isolated populations. Hum. Mol. Genet. 6, 2163–2172.

Collin, R.W., et al., 2011. High-resolution homozygosity mapping is a powerful tool to detect novel mutations causative of autosomal recessive RP in the Dutch population. Invest. Ophthalmol. Vis. Sci. 52, 2227–2239.

Daiger, S.P., Bowne, S.J., Sullivan, L.S., 2007. Perspective on genes and mutations causing retinitis pigmentosa. Arch. Ophthalmol. 125, 151–158.

den Hollander, A.I., Black, A., Bennett, J., Cremers, F.P., 2010. Lighting a candle in the dark: advances in genetics and gene therapy of recessive retinal dystrophies. J. Clin. Invest. 9, 3042–3053.

Ducroq, D., Shalev, S., Habib, A., Munnich, A., Kaplan, J., Rozet, J.M., 2006. Three different ABCA4 mutations in the same large family with several consanguineous loops affected with autosomal recessive cone-rod dystrophy. Eur. J. Hum. Genet. 14, 1269–1273.

Ferrari, S., Di Iorio, E., Barbaro, V., Ponzin, D., Sorrentino, F.S., Parmeggiani, F., 2011. Retinitis pigmentosa: genes and disease mechanisms. Curr. Genomics 12, 238–249.

Fingert, J.H., et al., 2008. Association of a novel mutation in the retinol dehydrogenase 12 (RDH12) gene with autosomal dominant retinitis pigmentosa. Arch. Ophthalmol. 126, 1301–1307.

Haeseleer, F., et al., 2002. Dual-substrate specificity short chain retinol dehydrogenases from the vertebrate retina. J. Biol. Chem. 277, 45537–45546.

Hartong, D.T., Berson, E.L., Dryja, T.P., 2006. Retinitis pigmentosa. Lancet 368, 1795–1809.

Janecke, A.R., et al., 2004. Mutations in RDH12 encoding a photoreceptor cell retinol dehydrogenase cause childhood-onset severe retinal dystrophy. Nat. Genet. 36, 850–854.

Khateb, S., et al., 2012. Exome sequencing identifies a founder frameshift mutation in an alternative exon of USH1C as the cause of autosomal recessive retinitis pigmentosa with late-onset hearing loss. PLoS One 7, e51566.

Mackay, D.S., et al., 2011. RDH12 retinopathy: novel mutations and phenotypic description. Mol. Vis. 17, 2706–2716.

Miano, M.G., Jacobson, S.G., Carothers, A., Hanson, I., Teague, P., 2000. Pitfalls in homozygosity mapping. Am. J. Hum. Genet. 67, 1348–1351.

Ng, S.B., et al., 2010. Exome sequencing identifies the cause of a Mendelian disorder. Nat. Genet. 42, 30–35.

Perrault, I., et al., 2004. Retinal dehydrogenase 12 (RDH12) mutations in leber congenital amaurosis. Am. J. Hum. Genet. 75, 639–646.

Schuster, A., et al., 2007. The phenotype of early-onset retinal degeneration in persons with RDH12 mutations. Invest. Ophthalmol. Vis. Sci. 48, 1824–1831.

Seelow, D., Schwarz, J.M., Schuelke, M., 2008. GeneDistiller — distilling candidate genes from linkage intervals. PLoS One 3, e3874.

Seelow, D., Schuelke, M., Hildebrandt, F., Nürnberg, P., 2009. HomozygosityMapper — an interactive approach to homozygosity mapping. Nucleic Acids Res. 37, W593–W599.

Sun, W., et al., 2007. Novel RDH12 mutations associated with Leber congenital amaurosis and cone-rod dystrophy: biochemical and clinical evaluations. Vision Res. 47, 2055–2066.

Thompson, D.A., et al., 2005. Retinal degeneration associated with RDH12 mutations results from decreased 11-cis retinal synthesis due to disruption of the visual cycle. Hum. Mol. Genet. 14, 3865–3875.

Tucker, B.A., et al., 2011. Exome sequencing and analysis of induced pluripotent stem cells identify the cilia-related gene male germ cell-associated kinase (MAK) as a cause of retinitis pigmentosa. Proc. Natl. Acad. Sci. U. S. A. 108, E569–E576.

Valverde, D., Pereiro, I., Vallespín, E., Ayuso, C., Borrego, S., Baiget, M., 2009. Complexity of phenotype–genotype correlations in Spanish patients with RDH12 mutations. Invest. Ophthalmol. Vis. Sci. 50, 1065–1068.

Wang, Y., et al., 2012. Exome sequencing identifies compound heterozygous mutations in CYP4V2 in a pedigree with retinitis pigmentosa. PLoS One 7, e33673.

Zenteno, J.C., Buentello, V.B., Ayala, R.R., Villanueva, M.C., 2011. Homozygosity mapping identifies the Crumbs homologue 1 (Crb1) gene as responsible for a recessive syndrome of retinitis pigmentosa and nanophthalmos. Am. J. Med. Genet. A 155A, 1001–1006.

Zlotogora, J., 2007. Multiple mutations responsible for frequent genetic diseases in isolated populations. Eur. J. Hum. Genet. 15, 272–278.

Züchner, S., et al., 2011. Whole-exome sequencing links a variant in DHDDS to retinitis pigmentosa. Am. J. Hum. Genet. 88, 201–206.

Taylor & Francis
Taylor & Francis Group

Expert Review of Molecular Diagnostics

ISSN: 1473-7159 (Print) 1744-8352 (Online) Journal homepage: http://www.tandfonline.com/loi/iero20

The clinical implications of molecular monitoring and analyses of inherited retinal diseases

Oscar F. Chacón-Camacho, Leopoldo A. García-Montaño & Juan C Zenteno

To cite this article: Oscar F. Chacón-Camacho, Leopoldo A. García-Montaño & Juan C Zenteno (2017) The clinical implications of molecular monitoring and analyses of inherited retinal diseases, Expert Review of Molecular Diagnostics, 17:11, 1009-1021, DOI: 10.1080/14737159.2017.1384314

To link to this article: http://dx.doi.org/10.1080/14737159.2017.1384314

Accepted author version posted online: 25 Sep 2017.
Published online: 05 Oct 2017.

EXPERT REVIEW OF MOLECULAR DIAGNOSTICS, 2017
VOL. 17, NO. 11, 1009–1021
https://doi.org/10.1080/14737159.2017.1384314

Taylor & Francis
Taylor & Francis Group

REVIEW

The clinical implications of molecular monitoring and analyses of inherited retinal diseases

Oscar F. Chacón-Camacho[a], Leopoldo A. García-Montaño[a] and Juan C Zenteno[a,b]

[a]Genetics Department-research Unit, Institute of Ophthalmology 'Conde de Valenciana', Mexico City, Mexico; [b]Biochemistry Department, Faculty of Medicine, UNAM, Mexico City, Mexico

ABSTRACT

Introduction: Retinal dystrophies (RDs) are the most common cause of inherited blindness and one of the most genetically heterogeneous human diseases. RDs arise from mutations in genes involved in development and function of photoreceptors or other retinal cells. Identification of the genetic defect causing RD allows accurate diagnosis, prognosis, and counseling in affected patients. Molecular diagnosis is a tremendous challenge in RDs due to their locus and phenotypic heterogeneity. As conventional DNA sequencing approaches are impractical in such situation, Next Generation Sequencing (NGS)-based protocols are needed to identify RD-causing mutations. This is being accomplished by sequencing RD gene panels or by whole exome or whole genome sequencing approaches.

Areas covered: This review discusses the current strategies for molecular diagnosis in RDs including their advantages and limitations, as well as their utility in diagnosis of non-syndromic versus syndromic RDs. Results of ongoing gene therapy protocols in RDs are also presented.

Expert commentary: Molecular diagnosis in RD improves the medical management of patients. Importantly, demand for molecular screening for RDs is greatly expanding not only as a result of increasing development and availability of NGS technologies, but also of the growing number of gene-based clinical trials offering a potential treatment to patients.

ARTICLE HISTORY
Received 29 June 2017
Accepted 21 September 2017

KEYWORDS
Retinal dystrophies; molecular diagnosis; next generation sequencing; retinitis pigmentosa; exome sequencing

1. Introduction

The application of molecular analysis for the diagnosis of genetic diseases has allowed unprecedented diagnostic precision in medicine, greatly favoring individualized care of patients and improving prevention of complications. For single gene disorders with a recognized clinical indication and/or known mutational hot spots, Sanger sequencing of the target gene or region has been traditionally used as an accurate and cost-effective method to reach a definitive molecular diagnosis [1,2]. Contrarily, molecular diagnosis in diseases with genetic heterogeneity (i.e. identical phenotypes being caused by mutations in one of dozens of genes) is a complicated task [3,4]. In this group of diseases, genetic diagnosis could take considerable amount of time, months or even years, using standard procedures that include PCR amplification and direct Sanger sequencing of candidate gene(s).

Currently, the choice of the gene to be analyzed for molecular diagnosis in genetically heterogeneous diseases relies on several criteria that includes testing first the most commonly mutated gene(s), screening of the gene according to phenotypic particularities (genotype-phenotype correlations), and previous knowledge of founder mutation effect in a given ethnic group, among other considerations [5,6]. If no causal mutations are identified after this approach, genetic analysis must be extended to other gene(s). However, mutation screening in a gene-by-gene analysis approach is time-consuming and expensive. Despite the inherent difficulties, molecular diagnosis of patients with suspected genetic diseases provides a number of benefits as it can supply an accurate prognosis of the clinical course of the disease and appropriate genetic counseling to the families for reproductive planning, including carrier testing and preimplantation or prenatal genetic screening. In addition, the molecular characterization of patients allows their future inclusion in clinical trials based on gene therapy.

Retinal dystrophies (RDs) are the most common cause of inherited blindness worldwide. They are defined as a group of disorders arising from genetic defects in proteins with a role in development, function, and maintenance of specific retinal cells [7]. RDs occur at a collective frequency of approximately 1 in 2,000–2,500 with retinitis pigmentosa (RP), affecting >2 million people worldwide, being the most common subtype [8,9]. Clinical symptoms, age of onset, and progression rate of RDs are extremely variable and depend primarily on the involved cell layer [10,11]. For example, RP (also known as rod-cone dystrophy) affects primarily rod photoreceptors and is characterized by nyctalopia, reduced peripheral visual fields, and typical pigmentary changes in retinal fundus. Conversely, cone-rod dystrophies, macular dystrophies, and pattern dystrophies exhibit predominantly cone (macular) dysfunction leading to decreased visual acuity, blurred vision, and color vision deficiency as initial symptoms. In cone dystrophies,

CONTACT Juan C Zenteno jczenteno@institutodeoftalmologia.org Genetics Department-Research Unit, Institute of Ophthalmology 'Conde de Valenciana', Mexico city, Mexico

there is clinical (dyschromatopsia, central scotomata, nystagmus and photophobia) and electrophysiological evidence of abnormal cone function [12]. The rare stationary forms of cone dystrophy showing no rod involvement in advanced stages are termed cone dysfunction syndromes [13]. Isolated dysfunction of rods is observed in congenital stationary night blindness (CSNB), a non-progressive disorder manifesting as night or dim light vision disturbance or delayed dark adaptation [14].

Although most RD cases initially exhibit localized affectation to a specific cell type, it is extremely common that other retinal layers be affected in advanced stages of diseases, which produces aggravation of visual deficiency and eventually, blindness. In late stages of disease, phenotypic characterization of RD is difficult as the entire retina might be affected [15,16].

RDs are one of the most genetically heterogeneous diseases in humans. Since the identification of Rhodopsin as the first RD gene in 1990 [17], approximately 250 genes responsible for isolated (non-syndromic) or syndromic forms of the disease have been characterized to date (https://sph.uth.edu/retnet/, accesed on 1 June 2017). RD-associated genes exhibit all Mendelian modes of transmission (autosomal recessive, autosomal dominant, and X-linked) and some of them are even transmitted atypically as mitochondrial or digenic traits [10,18]. Remarkably, proteins encoded by known RD genes have roles in a strikingly variety of biological processes including retinoid recycling pathway, function and maintenance of the connecting cilium, phototransduction cascade, RNA splicing, retinal development, components of the cytoskeleton and the extracellular matrix, and lipid metabolism, among other pathways [19,20]. At present, it is not completely understood why defects in proteins needed for essential cellular roles manifest only as retinal cell degeneration.

The remarkable locus heterogeneity of RDs is not the only factor complicating an efficient molecular diagnosis in affected patients. Also, mutations in the same gene can produce different retinal phenotypes, both among subjects from different families and between patients from a same pedigree [21,22]. For example, mutations in *CRX* (MIM 602225), encoding a master regulator of photoreceptor gene expression, may result in a range of dissimilar retinal phenotypes including cone-rod dystrophy, Leber congenital amaurosis (LCA), RP, and cone dystrophy [23]. Genetic defects in the *GUCY2D* gene (MIM 600179), encoding a retina-specific guanylate cyclase predominantly expressed in photoreceptors, are responsible for dominant forms of cone dystrophy or cone-rod dystrophy, but also for cases of recessively inherited LCA and RP [24,25]. Atypical patterns of inheritance can also occur as observed in families carrying genetic defects in *PRPF31* (MIM 606419), which codes for a protein involved in pre-mRNA splicing [26]. Dominant mutations in *PRPF31*, accounting for up to 10% of autosomal dominant RP cases, can be non-penetrant in some families [27], implicating that additional genetic variants allow the phenotypic expression of particular mutations (epistatic effect) [28].

To complicate the picture, it is estimated that 30–40% of genes involved in RDs are currently unknown [29,30], and thus, a molecular cause in a subset of individuals cannot be demonstrated even if all currently known RD genes are analyzed. For RP, the most common RD subtype, nearly 90 genes have been recognized to date [31].

2. NGS technologies for molecular characterization of RDs

NGS technologies have revolutionized molecular diagnosis by allowing the simultaneous sequencing of thousands of genes or even whole genomes in a single experiment [32,33]. This approach has demonstrated to be tremendously effective in identifying the underlying cause of monogenic diseases with no specific clinical diagnosis or in diseases with genetic heterogeneity including neurologic, cardiac, skeletal, and intellectual disorders, among many others. Given the extreme locus heterogeneity of RDs (~250 characterized loci to date), NGS has emerged as a cost-effective approach for the identification of mutations causing this expanding group of inherited diseases [34,35]. NGS can be applied to characterize all variants harbored by the whole group or a subgroup of RD genes (gene panel sequencing), sequencing the portion of the genome (~1%) encoding for proteins (exome sequencing) or even for sequencing the entire patient's genome (whole genome sequencing (WGS)) [36].

2.1. Sequencing of gene panels

NGS gene panel approach involves the sequencing of genes that have been targeted and validated for a specific disease or group of related diseases. This characteristic can make gene panels much more sensitive than other sequencing approaches, such as whole exome sequencing (WES). Recently it has been shown that gene panels have similar sensitivity compared to whole genome sequencing (WGS), when identifying pathogenic variants [37]. Using disease-specific gene panels may allow to sequence more samples per run, increasing read depth and coverage of screened regions at a cheaper cost. Because fewer genes are sequenced when gene panels are used, fewer disease-unrelated variants are identified, therefore, reducing the risk of secondary findings [38,39] and decreasing the chances of calling variants of uncertain significance or non-pathogenic variants that could lead to an uncertain diagnosis. Still, one of the greatest advantages that gene panels offer is the possibility to personalize their design with any coding or non-coding region within a genome.

2.2. Gene panel sequencing for molecular diagnosis of RDs

RDs are excellent candidates to be screened through gene panels due to their wide genetic heterogeneity and clinical phenotypic variability. Several research groups have designed distinct panels using a variable combination of genes with the intention to ease variant detection in RD patients. Reported rates of success in the discovery of causal variants with these panels ranges from 50% to 76%, simultaneously allowing the characterization of novel pathogenic variants in several known RD genes [37,40–44]. Additionally, commercial companies such as Illumina and Agilent, offer pre-designed panels that

contain thousands of clinically relevant genes, including most of the genes known to be implicated in RDs. For example, the Illumina Trusight One kit utilizes a panel designed for the enrichment of 4,811 genes involved in human inherited diseases, including 197 out of the 256 genes that, as in May of 2017, have been identified to be associated with inherited retinal diseases (https://sph.uth.edu/retnet/). Similarly, the SureSelect Inherited Disease panel, developed by Agilent, includes probes that allow for the capture of the coding regions of 2,742 genes related to inherited diseases, of which, 193 are RD genes. Other groups have used a more specific enrichment approach for screening large genomic regions including a particular locus, as recently shown for *ABCA4* (MIM 601691) in Stargardt disease (MIM 248200) [45]. This approach resulted in increased variant sensitivity and discovery rate by including the analysis of both exonic and intronic sequences [45]. A*BCA4* is a highly polymorphic gene comprised of 50 exons and 49 introns. Because of its great size, direct Sanger sequencing is neither practical, nor economically convenient, and an NGS approach for this gene is likely to be less expensive and time consuming for its proper analysis [46].

2.3. Considerations and limitations of gene panel sequencing in the analysis of RDs

One of the most important aspects to consider when using gene panels for NGS is the enrichment method used to select the regions to be sequenced. The most common enrichment approaches include amplicon-based enrichment, which relies mainly on amplification of certain regions of the genome, and a combination of PCR and hybridization enrichment probes. Former work has shown that capture methods using hybridization probes have a more efficient performance than amplicon-based methods when sequencing and creating datasets to call true genetic variants [47]. Recently, capture methods developed by Agilent, Illumina, and NimbleGen were compared in a study that analyzed 24 patients with a custom panel designed to search for mutations in genes associated with non-syndromic hearing loss, Usher syndrome, and autosomal recessive RP. It was observed that, even though they show significant differences in their performance, all three technologies proved to be effective in building datasets reliable and useful for variant detection [48]. However, it is important to consider that this effectiveness and reliability may depend on certain parameters and quality standards when building libraries and analyzing results. Some of these parameters include read depth and coverage of each sample during the sequencing run, the amount of reliable data produced, and the pipelines used to manage data and to call true gene variants. Therefore, despite the fact that current technologies have demonstrated efficiency to detect pathogenic variants in patients with retinal diseases, some of this effectiveness also relies on a variety of technical aspects. Furthermore, because most approaches to enrich gene panels are based on PCR amplification, they may be accompanied by some limitations inherent to this technique. Some of these limitations may include low coverage (or no coverage) of regions intractable by PCR amplification, such as GC-, AG-rich regions, or the untranslated regions of many genes [49]. Additionally, because most gene panels are represented by only a subset of targeted coding regions, most of the times they fail to reveal emerging and novel causes for RD, such as pathogenic variants found in introns or in genes that have not been associated to the disease before, and do not produce appropriate data for the analysis of structural genomic rearrangements [40].

2.4. Whole exome sequencing (WES)

Even though gene panels have shown to be useful for reaching a molecular diagnosis in RD patients, this may not always be the case. Sometimes, we might find ourselves with incomplete results or no conclusive results at all (i.e. no causal mutation(s) identified) after analyzing data obtained from custom gene panels sequencing. Whenever personalized panels fail to detect the pathogenic variants responsible for a particular phenotype, exome sequencing may be considered as an option. WES involves the enrichment and sequencing of all protein-coding nuclear genes within a genome (not including mitochondrial genes). Most exon-enrichment approaches are very similar to the ones used to generate gene panels and involve a combination of PCR and hybridization enrichment probes. Approximately, 2% of human genome is composed by exons and ~95% of the exome can be sequenced with the currently available techniques [50]. Therefore, unlike gene panels, WES offers the possibility to discover novel RD-associated genes. For example, a WES study performed by De Castro-Miró et al. [51] analyzing 33 families with a variety of retinal disorders identified *SEMA6B (MIM 608873), CEP78 (MIM 617110)*, and *SCLT1* (MIM 611399) genes as novel candidates for non-syndromic RDs. Although success rates vary considerably according to the type of disease and sample characteristics, it has been shown that WES can effectively lead to a reliable diagnosis in about 20–30% of the cases where the patient had been previously undiagnosed [39]. However, because WES enrichment methods are also based on PCR they may share some limitations with gene panels, such as uneven coverage along certain regions of the genome, especially GC-, AG-rich regions, and off-target enrichment [49].

2.5. Whole genome sequencing

WGS is a free-PCR NGS approach that refers to the sequencing of all the coding (exons) and non-coding (introns, regulatory, and intergenic sequences) regions within a genome. Because of this characteristic, WGS has proved itself a very efficient tool for the discovery of new variants, especially those located in non-coding regions or involving structural rearrangements that are not easily detectable by gene panels or WES. A study made by Ellingford et al. in 2016 [40] compared the efficacy of WGS in diagnosing inherited RDs in relation to gene panels and concluded that, even though WGS and gene panel sequencing shared similar sensitivity and specificity, the use of WGS pipelines could have a higher detection rate of pathogenic variants than gene panels, increasing the diagnostic yield up to 29% more. Additionally, WGS showed to have a wider spectrum for variant detection by identifying large deletions that ranged from 520 kb to 1.7 kb, mutations in non-coding regions excluded from the targeted sequencing pipeline, and pathogenic variants in genes that were not included

in sequencing panels [40]. Likewise, Carrs et al. [36] recently showed that WGS could uplift diagnostic yields resulting from WES by up to 30%, reaffirming WGS ability to identify structural variants and mutations that are not covered either by gene panels or WES. This approach has also been helpful for understanding the nature of some specific forms of RD. For example, a recent WGS analysis in patients from three different families suffering from an RD known as North Carolina Macular Dystrophy (MIM 136550) allowed for the identification of rare non-coding causal variants [52].

Aside all the benefits, it has been difficult for most laboratories around the world to adopt WGS as a routine test because it still remains expensive and more time consuming compared to WES and gene panels, and it involves the need of great computer power and storage for handling WGS processing and data. Analyzing the entire data produced by WGS might seem as an overwhelming idea. However, several work groups have developed and applied very useful bioinformatic tools and pipelines to generate virtual gene panels that aid in WES and WGS data analysis [53]. This approach can be used to extract variants from selected genomic regions from a data set resulting from WES or WGS, facilitating its analysis. As new variants responsible for the phenotypes of interest are being discovered, virtual gene panels can be updated for future analysis of the same datasets without the need to repeat experiments in order to reach a correct molecular diagnosis [54].

2.6. Important aspects to consider to have a reliable molecular diagnosis for RDs through NGS

One of the most important aspects to successfully obtain a reliable interpretation from molecular data in RD patients is, unquestionably, to have a specific pre-test clinical diagnosis. Because of the great clinical variability of RDs and the heterogeneity of genes associated to this group of diseases, the findings revealed by NGS may sometimes not be consistent with the initial clinical impression. Therefore, it is needed to call the patient for a revaluation, based on the genetic findings, in order to determine a correct diagnosis [51], an approach that has been termed 'reverse phenotyping' [55].

In some cases, even when the initial clinical diagnosis is believed to be accurate in RD patients, the rate of pathogenic variant detection obtained through gene panels may be low. In a recent study of 16 rod-cone RD patients of Maori and Polynesian descent using a 105 genes-specific panel for inherited retinal diseases, a 43.7% rate of molecular diagnosis was obtained [56]. This result could be due because the panel used in this study is only using about half of the genes reported to be implicated in RDs (https://sph.uth.edu/retnet/). Therefore, it is very important that gene panels be up to date with the most recently described RD genes in order to achieve a higher variant detection rate. These results also indicate that pathogenic variant detection may depend in some degree on specific characteristics of the population or ethnic group being evaluated [56]. If a specific population has pathogenic variants in novel or in recently described genes, it might be difficult to detect pathogenic variants through gene panels, and WES and WGS may be needed for a definitive molecular diagnosis.

An important issue when analyzing data generated by NGS is the validation of candidate pathogenic variant(s) by means of an alternative methodology. Currently, most laboratories include Sanger Sequencing as the 'golden standard' to validate NGS results. However, recent studies from different work groups have suggested that NGS results may be as reliable as Sanger sequencing data, and that Sanger validation, in many cases, is unnecessary. Most of these studies have used Sanger sequencing for screening groups of variants detected by gene panel sequencing or WES in subsets of genes resulting from targeted sequencing and WES, consistently observing validation rates above 99% [57–60]. A study by Beck et al. in 2016 even suggested that a single round of Sanger sequencing is more likely to incorrectly refute a true-positive variant from NGS than to correctly identify a false-positive variant from NGS [61]. These results have suggested that NGS data are reliable by itself and that Sanger validation could be redundant, time consuming, and unnecessary. However, it is important to consider that different enrichment methods for NGS libraries may vary in performance regarding read depth, coverage, and sequencing quality in general. For example, Samorodnitski et al. [47] suggested that amplicon-based methods were more likely to miss variants that were detected by hybridization-based approaches. Furthermore, as will be discussed later in more detail, NGS technologies still fail to completely produce reliable and reproducible data, as occurs for repetitive and GC, GA-rich regions of the genome, leaving Sanger sequencing as a better candidate for variant analysis in such cases. Overall, these studies imply that, if sufficient quality and control standards are applied during NGS analysis, Sanger sequencing may not be strictly necessary to validate NGS data, but it is still recommended for low-quality data and for certain regions of the genome that may be difficult to analyze through NGS methods. Of course, familial segregation analysis of the candidate causal variant(s) becomes an essential tool to support the pathogenicity of a mutation (especially when working with familial RD cases), and this is easily accomplished by means of targeted Sanger sequencing.

2.7. Limitations and challenges of NGS in molecular diagnosis of RDs

Even though gene enrichment panels may seem a convenient option to identify pathogenic variants and provide a fast and certain molecular diagnosis, they have some limitations. For example, if a gene has not been linked to a specific disease at the time of the panel design, it will escape detection. In some cases, even though the genes responsible for a disease have been included in a panel, the sensitivity of the enrichment method can prevent a certain region to be captured, and the disease-causing variants will only be detected by a more sensitive method [44]. As before mentioned, the effectiveness of a gene panel may also depend on technical aspects during library preparation and data analysis. Another disadvantage is that gene panels may only include genes that have been described or validated for a certain phenotype, preventing the possibility to detect variation in novel disease genes or in non-coding sequences. Additionally, some studies have suggested that the rate of success of a specific gene panel

may vary depending on the ethnic group being studied and that the variation sensitivity of a panel can drop significantly when working with very specific ethnic groups [56]. To overcome this point, several groups have developed ethnic-based gene panel as a recent one applied to RD patients from Spain [62]. It is also important to consider that even though NGS is an excellent option for genetic testing of RDs, they may show certain shortcomings. Some of the causal genetic mechanisms that cannot reliably be detected by clinical exome sequencing include alterations that influence gene expression without DNA modifications, such as imprinting errors and uniparental disomy, or causal variants being located in highly repetitive regions of DNA or in mitochondrial DNA. Additionally, NGS is not a reliable approach to detect somatic mosaic changes or, in case of targeted sequencing and WES, large copy number variants [63]. Furthermore, even though some studies report that WGS can significantly improve the coverage of repetitive regions compared to sequencing panels and WES [36], it still fails to generate consistent coverage across these areas of the genome. For example, in a recent study comparing targeted sequencing vs WGS, it was observed that neither approach was able to generate consistent coverage of the AG-rich, repetitive regions of *RPGR*orf15 (MIM 312610) [40], an *RPGR* retinal photoreceptor isoform, which is responsible for about 70–75% of all X-linked RP [64]. Therefore, Sanger sequencing remains as a pertinent method to detect genetic variants in such regions. A comparison of features among the three main NGS approaches (gene panels, whole exome sequencing, and whole genome sequencing) is presented in Table 1.

NGS is becoming the gold standard test for molecular diagnosis of RD patients, and it is expected the detection rate will increase in the next few years by improving genomic coverage and nucleotide read depth as well as by refinement of variant calling pipelines, and best classification of non-pathogenic variants, especially for candidate intronic or synonymous variants. In addition, the establishment of more precise genotype–phenotype correlations and a wider accessibility of genetic testing services will positively impact in a better molecular characterization of RDs.

3. Importance of molecular diagnosis in RDs

Extensive clinical heterogeneity and overlapping phenotypes in RDs frequently impede a specific diagnosis based on retinal clinical findings alone, and thus, genetic testing is essential for these patients. As previously mentioned, identification of the genetic defect that causes retinal disease has critical implications, not only in prognosis and genetic counseling in affected families, but also in the recognition of patients who could benefit for the increasing number of gene therapy protocols. On the other hand, it is expected that the wider availability of genetic counseling services and of molecular diagnostic platforms facilitates inclusion of RD patients in molecular diagnostics protocols. In a recent survey, Eden et al. [67] interviewed 48 patients with RP and showed that 92% of them desired genetic counseling and 86.5% wanted genetic testing, demonstrating that most patients value these services. While during the last three decades RDs genetic diagnosis has relied in Sanger sequencing, the advent of the NGS technology has opened an extraordinary opportunity for genetic characterization of RD patients. NGS studies for molecular diagnosis of RDs are available in a growing number of specialized care centers. Figure 1 shows a scheme representing the utility of NGS approaches considering the extensive phenotypic and genetic heterogeneity of RDs.

3.1. Syndromic vs non-syndromic RDs: impact of molecular diagnosis

RD can occur as an isolated anomaly or be accompanied of extraretinal findings and are classified as syndromic RD. Up to 30% of RP cases have associated findings and there exist at least 30 different syndromic forms of RP, with Usher syndrome (20%) and Bardet-Biedl syndrome (BBS) (6%) being the two most frequently diagnosed [68]. Systemic anomalies in syndromic RP are diverse and include dissimilar findings as hearing loss and vestibular dysfunction in Usher syndrome, obesity and polydactyly in BBS, and metabolic disturbances and cardiomyopathy in Alström syndrome, to cite a few examples. Since RD can be the first manifestation of a multisystemic condition, early diagnosis is crucial as some features of syndromic RDs are treatable or preventable. This is particularly important in young children in whom molecular testing can identify those patients who are at risk of systemic involvement, such as renal, neurologic, or metabolic anomalies, and who will benefit from opportune diagnosis and treatment [69]. Early identification of a syndromic RD allows parents and physicians to know what systemic complications may arise and therefore require periodical scrutiny for early detection. In young patients, diagnosis can be challenging, as characteristic clinical features of some syndromes (e.g. obesity, hearing loss, diabetes mellitus, cardiovascular anomalies, neurologic disturbances) may not be apparent until later childhood [16,70]. In sum, the impact on the health management of the patient will be completely different in syndromic versus non-syndromic RD.

As previously mentioned, molecular testing has direct implications in clinical diagnosis and prognosis of RD patients. Recognition of a genetic cause can allow identification of syndromic forms of RD not clinically suspected due to clinical variation among patients. For example, recessive mutations in the *CLN3* gene (*607042) have been classically associated with Batten disease (#204200, also known as Ceroid Neuronal lipofuscinosis), a severe neurological entity characterized by early onset RD (4–7 years of age) causing loss of visual acuity and leading to blindness within 2–10 years and followed by cognitive decline, behavioral and psychiatric disturbances, and epileptic seizures developing between 7 and 18 years of age [71]. The severe and progressive cognitive and motor decline leads to premature death at third decade of life. Recently, mutations in *CLN3* have been also demonstrated in adult individuals with apparently non-syndromic RD indicating that *CLN3* mutations are not invariably associated with the devastating Batten disease [72]. This variability has tremendous implications for genetic counseling and prognosis in affected families. Similarly, mutations in *ABHD12* (*613599) have been associated with PHARC syndrome, an autosomal recessive neurodegenerative disease that leads to demyelinating **P**olyneuropathy, **H**earing loss, cerebellar **A**taxia, **R**P, and early-onset **C**ataracts (PHARC, #612674) [73]. Recently,

Table 1. Comparison among different sequencing approaches: gene panel sequencing, whole exome sequencing and whole genome sequencing.

	Gene panel (targeted) sequencing	WES	WGS
Targeted regions	Targets-specific regions validated for a disease or group of diseases [39]	Targets only the coding regions of a genome [39]	Targets all exons and non-coding regions in a genome [39]
Sample preparation	Involves enrichment of specific regions using primers or probes [48]	Involves enrichment of exons through probes and/or PCR [47]	No need for enrichment strategies (PCR-free) [39]
Read depth and coverage	Generally increases read depth and coverage for specific genes (depending on the design), making it more sensitive than WES [37], with similar sensitivity than WGS [40]	95% of exome regions at 20x. [65]	Studies report up to 98% of human genome at 87x [65]. Generally, it involves more even coverage than enrichment methods [40]
Cost	Cheaper than WES or WGS; very accessible for most laboratories [39]	Moderate price; accessible for laboratories [39]	Significantly higher than WES; not very accessible for most laboratories [39]
Allows discovery of new variants in genes already reported for a specific disease?	Yes (if the gene is included in the panel) [40–44]	Yes [51]	Yes [36]
Allows discovery of new variants in novel genes for a specific disease?	Not likely (unless gene is included in the panel design)	Yes [51]	Yes [36]
Allows for the discovery of deep intronic variants?	Not unless the intronic region is included in the panel design [45]	Not likely, only in flanking regions of coding sequences	Yes [36,52]
Allows detection of variants in repetitive, GC-AG-rich regions?	Yes [40], although variations in read depth may lead to false positive results [39]	Yes [36] but shows great variations in coverage. Read depth may lead to false positive results [39]	Yes [40] Shows improved coverage compared to targeted sequencing and WES, but variations in read depth may lead to false positive results [39]
Allows detection of CNVs?	Not likely [40]	Yes [36], but variations in read depth may lead to false positive results [39]	Yes, it shows improved coverage compared to targeted sequencing [40] and WES [36]
Diagnostic utility	Varies depending on the panel design and disease (50–76% reported for RD patients) [40–44]	20–30% of previously undiagnosed individuals [66]	May uplift result yields by ~30% compared to targeted NGS [40] or exome sequencing [36]
Is there probability for secondary (Incidental) findings	Not likely (Unless different genes are included in the panel design) [38,39]	Yes [38]	Yes [38]
Includes mitochondrial sequences?	May include mitochondrial sequences (if included in the panel design) [39]	Does not include mitochondrial sequences [39]	Includes mitochondrial sequences [39]

Nishiguchi et al. [74] demonstrated that *ABHD12* mutations can also result in non-syndromic forms of retinal degeneration implying that PHARC syndrome can exhibit extreme clinical variability, even within a pedigree [74]. These observations have profound implications in the differential diagnosis of 'deaf-blindness' diseases (including Usher syndrome) as confirming the presence of associated symptoms is necessary for differentiating some deaf-blindness syndromes. Again, mutation analysis is a central tool for confirming the diagnosis. In another example, mutations in *IFT172* (*607386), a member of a gene family encoding proteins involved in the process of intraflagellar transport, are a recognized cause of Bardet-Biedl syndrome, a syndromic form of RD which also exhibits polydactyly, obesity, genital abnormalities, renal defects, and learning difficulties (OMIM PS209900) [75]. Bujakowska et al. [76] showed that *IFT172* mutations can result in isolated RD forms (RP71; #616394), which indicates that mutations in genes originally reported to be associated with syndromic ciliopathies should also be considered in subjects with non-syndromic RD. These few examples illustrate that although it is complex to establish a definitive genotype–phenotype correlation in RD patients, NGS analysis is rapidly establishing the spectrum of associated phenotypes linked to mutations in RD genes.

In young patients, RD can be the earliest manifestation of a syndromic entity, and thus, the patient be erroneously diagnosed as having an isolated form of disease. In such cases, sequencing of panels that only includes genes known to be mutated in isolated forms of retinal disease might result in diagnostic failure. Therefore, a patient with RD that tested negative for a gene panel specific for isolated RDs, should also be considered to be analyzed for panels containing genes involved in syndromic RDs. Choosing a panel that contains genes responsible for syndromic and non-syndromic RDs (or even exome sequencing) could be a better initial approach, particularly in centers offering genetic testing to pediatric population.

4. Gene therapy in RDs: the role of molecular analysis

A major impact field of molecular diagnosis of RDs is the identification of patients who carry genetic defects that are amenable to be treated through gene therapy approaches. Although there are few available gene therapy protocols for RDs, an increasing number of preclinical trials are being conducted for a number of other subtypes of the disease [77]. In the following section, we will revise the main clinical results of ongoing gene therapy protocols for RDs.

Gene therapy is an approach intended to treat, cure, or prevent a disease by transferring one or more therapeutics nucleic acids to patient's cells (gene replacement therapy) or by correcting a defective gene (elimination of mutant alleles). The eye, an immunologically privileged, well compartmentalized, and easy accessible monitoring organ, is an ideal target for new genetic therapies. The use of different genetic vectors with minor unfavorable effects, the availability of comparative studies using eyes of large animal models, as well as the possibility of using the contralateral untreated eye as control are other features that make the eye a suitable organ for gene therapy [78,79].

4.1. Human gene therapy in LCA due to *RPE65* mutations

Gene transfer for restoring vision in an RD subtype named Leber congenital amaurosis or LCA is one of the most successful examples of gene therapy for a human disease. LCA is a congenital RD that affects individuals since very early ages and comprises a spectrum of retinal phenotypes with considerable variability in disease progression. Early and severe visual deficit before one year of age, severely subnormal or non-detectable ERG, and associated signs as nystagmus and near absent pupillary response are the most common LCA clinical findings [80]. At least 22 genes associated to LCA have been identified to date, with autosomal recessive transmission in virtually all of them [81]. Mutations in *RPE65* (*180069) are responsible for LCA2 (#204100), a subtype of the disease accounting for up to 15% of all LCA forms [55]. In 2008, three independent studies began phase I clinical trials in order to assess the efficacy of unilateral subretinal injection of the AAV-RPE65 vector in LCA patients carrying *RPE65* mutations. Bainbridge et al. [82] included three young adults with early-onset, severe retinal dystrophy with limited residual retinal function. In each subject, the worse eye was selected for the gene therapy. Microperimetry, dark-adapted perimetry, and visual mobility time in low light improved in one patient. Likewise, mobility errors decreased from 8 to 0 in the same patient (Bainbridge et al., 2008). Maguire et al. [83] studied three LCA patients with ages between 19 and 26 years, in whom a transgene carrying the human RPE65 cDNA was retinally injected. Baseline testing of pupillary light reflex in all patients shown much less sensitivity to light than control subjects. Two weeks after the procedure all patients also demonstrated improvement of vision in dimly environments and several months after, the eyes that received the injection became almost three times as sensitive to light when compared to baseline. All patients presented severe nystagmus at baseline, which reduced after the gene therapy. Hauswirth et al. [84] reported an independent clinical trial in which an rAAV vector gene therapy was subretinally injected to the eye with worse vision in three young adults (aged 21–24 years) suffering from *RPE65*-related LCA. Although visual acuity did not show significant difference from baseline, all patients showed increased light sensitivity which was more perceptible in decreased light conditions [84].

In 2009, Maguire et al. [85] presented results for the complete phase 1 dose-escalation trial, which assess the safety and efficacy of AAV2. Twelve patients were recruited and treated with AAV2 in the eye with worst vision. Patients were treated in three different cohorts depending on the dose of vector: low, medium, and high doses. All patients showed improved vision in dimly lit environments as early as 2 weeks after injection. Improvements in visual acuity were substantial in all patients who received both low and middle doses, as well as one patient administered in high dose. Visual field improvement was observed in all cases while improvements in full-field sensitivity were noteworthy in youngest patients. Pupillary responses improvement occurred in all individuals tested, and was detectable as early as day 7 after injection. After administration, four children presented great improvement in their ambulation when using only their treated eye

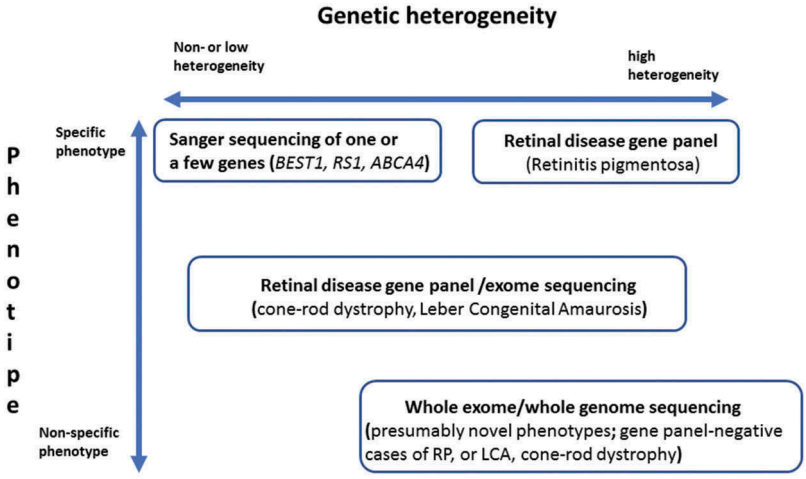

Figure 1. A scheme representing the utility of different sequencing approaches based on phenotypic specificity and genetic heterogeneity in RDs.

[85]. In 2015, Bainbridge et al. [86] reported three-year results of a phase 1–2 human *RPE65* trial, involving 12 participants to evaluate the safety and efficacy of the gene therapy. Improvements in retinal sensitivity were seen in six participants on dark adapted perimetry and in five of these participant on microperimetry. Five participants displayed improvements in night vision, and three of them showed improvements in vision-guided ambulatory navigation [86]. After the success of gene therapy in one eye, Bennet et al. [87] investigated if retinal and visual function may improve if contralateral eye, the better-seeing eye, is injected with AAV2-RPE65 vector. Twelve patients were enrolled (11 of them from the original study) and were followed up at the Children´s Hospital of Philadelphia. In four patients, visual field showed expansions by day 30 after the initial injection, and there were also robust improvements in both rod and cone function by day 30 in the contralateral eyes. Improvements from baseline remained during the 3 years analyzed. Most patients improved mobility after administration of contralateral eye [87].

In addition, Spark Therapeutics recently reported positive phase III results for SPK-RPE65 targeting the treatment of LCA2 by *RPE65* gene mutations. The phase III trial enrolled 31 patients, who improved navigation on the mobility test, with highly significant differences ($p = 0.001$) in 21 patients when compared with the control group (10 patients). Treated patients also outperformed control subjects in other key secondary endpoints as full-field light sensitivity threshold testing ($p < 0.001$) and the mobility test score change for the first injected eye ($p = 0.001$). Interestingly, 13 of 20 treated patients passed the mobility test at the lowest illumination level of one lux (equivalent to a moonless summer night), while control (uninjected) patients did not pass this test during one year. With this positive phase III results, Spark Therapeutics has requested an FDA Biologics License Application and also submitted a Marketing Authorization Application (MAA) to the European Medicines Agency (EMA) for the *RPE65* gene therapy treatment [88,89].

4.2. Gene therapy for other RDs

Choroideremia (#303100) is an X-linked recessive, gradually progressive, and degenerative retinal disease mainly affecting males and arising from mutations in *CHM1* (*300390), a gene located in Xq21.2 [90]. Its cardinal findings are reduced night vision in adolescence associated with loss of peripheral vision and blindness in middle age. A Phase I/II clinical trial of subretinally delivered CHM in six patients with choroideremia was recently completed by MacLaren et al. [91], showing the therapy to be safe. Six male patients at different stages of the disease were enrolled and all of them had a mean gain in visual acuity in their treated eyes of 3–8 letters. The point of maximal light sensitivity increased 2–3 dB in the treated eye 6 months after surgery. At 6-month follow-up, microperimetry showed reduction in retinal sensitivity of −0.8dB for the untreated eyes when compared with treated eyes, while in the treated eyes a 2.3 dB increase was noticed [91]. One third of the patients in the treatment group had more than 15-letter improvement at 3.5 years compared with none in the control group [92].

Stargardt disease (#248200) is an inherited macular dystrophy caused by biallelic mutations in *ABCA4* (*601691), a gene coding for a retinal transporter protein [46]. Stargardt disease is the most common cause of macular degeneration in pediatric ages and patients with the disease suffer from severe and irreversible vision loss within their first or second decades of life. Conservation of photoreceptor cells until adult life could permit sufficient time for carrying out a gene transfer therapy [93]. A clinical trial for patients with Stargardt disease (NCT01367444; www.clinicaltrials.gov) for subretinal delivering of ABCA4 using StarGen, a lentiviral viral vector, started in 2011. The study is being conducted in the United States and France. It is currently in phase II/III with 46 enrolled patients, and it has been estimated to be completed by November 2018. Although results have not been published yet, in 2012 Oxford BioMedica and Sanofi informed a Data Safety Monitoring Board evaluation indicating that eight patients were treated at dose level 1, and no serious adverse events related to StarGen or its methods of administration were observed. Twenty-eight new patients were enrolled to evaluated three dose levels for safety, tolerability, and aspects of biological activity (http://www.oxfordbiomedica.co.uk/press-releases/oxford-biomedica-announces-positive-dsmb-review-of-ongoing-retinostat-r-and-stargen-clinical-studies/).

MERTK (*604705), a gene encoding a protein involved in the phagocytosis regulation of the retinal pigment epithelium, is

mutated in about 3% of RP cases [94]. In a study published in 2001, it was shown that subretinal gene transfer of *Mertk* to the retina of the Royal College of Surgeon (RCS) rat, a well characterized model of recessive RP arising from a mutation in the *Mertk* gene, resulted in correction of the RPE phagocytosis defect and preservation of photoreceptors [95]. More recently, LaVail et al. [96] demonstrated that when the RPE-specific AAV2-VMD2-hMERTK vector was injected subretinally, it also protected photoreceptors from degeneration and reversed the phagocytic defects in the RCS rat model. They also found that in the Mertk knockout mouse, which exhibits rapid loss of most photoreceptors, subretinal injection of the AAV2-VMD2-hMERTK vector protected a majority of photoreceptor cells from degenerating. Results of a phase I clinical trial of an AAV2 vector expressing human MERTK cDNA driven by an RPE-specific promoter in patients with MERTK-associated retinal disease (NCT01482195) were recently published (Ghazi et al. 2016). Six patients aged from 14 to 54 years and with baseline visual acuities ranging between 20/50 and less than 20/6,400, were followed for up to 2 years after treatment. At the 2-year time point, none of the patients developed complications that could be attributed to the gene vector. Three patients had measurable improvements in visual acuity (VA); for instance, patient 1 had a preoperative VA of 20/6400 at a testing distance of 0.5 m, and his postoperative examination at days 10, 30, 90, and 365, were 20/160 (0.5m), 20/80(0.5m), 20/40 (0.5m), 20/50(0.5m), respectively. Unfortunately, by 1.5 years, and 2 years after injection, a marked VA reduction was noted. Patients in this cohort suffer from advanced RP (RP38) and it will probably be necessary to evaluate the true efficacy of the *MERTK* gene therapy in patients with better visual acuities [97].

Finally, other clinical trials for gene therapy in inherited RD are being conducted in diseases as achromatopsia (#262300; #216900) caused by *CNGB3* (*605080) or *CNGA3* (*600053) mutations (https://clinicaltrials.gov/ct2/show/NCT02599922 and NCT02610582), Usher syndrome type 1B (#276900) due to *MYO7A* (*276903) defects (https://clinicaltrials.gov/ct2/show/NCT01505062), and X-linked retinoschisis (#312700) caused by *RS1* (*300839) mutations. (https://clinicaltrials.gov/ct2/show/NCT02416622 and CT02317887).

Although limited, available data indicate that gene therapy protocols for RDs stand as an option for preventing blindness in affected individuals. Again, a prerequisite for enrolling patients in gene therapy trials is the characterization of the causal mutation.

5. Stem cell therapy for inherited retinal diseases

Stem cells have the ability to differentiate into several cell types but they are also capable of indefinite self-renewal in their undifferentiated state. Considerable developments have made over the past few years on the therapeutic application of stem cells, particularly in the form of cell replacement therapy. Here, the stem cells are differentiated into the desired cell type, which is then delivered to the damaged tissue in order to integrate and restore function. This therapy is an attractive option for cell replacement in retina, and to date approximately 25 stem/progenitor cell therapy trials are listed on www.clinicaltrials.gov. Three different sources of these cells can be used: pluripotent Stem Cells (PSC), fetal, and postnatal [98]. At present, two types of PSCs are used for the treatment in retinal degenerations: human embryonic stem cells (hESCs) [99], and induced pluripotent stem cells (iPSCs) [100]. ESCs are isolated from developing inner cell mass of blastocyst during 4–5 days after fertilization and prior to implantation [98], while iPSCs are pluripotent cells derived from adult cells that can be generated by viral transduction of a variety of transcription factors including Oct4, Sox2, Klf4, and Myc [101,102]. One potential advantage of iPSC-derived retinal precursor cells is the possibility to be originated from the same patient to whom the transplant is destined, thus decreasing the risk the immune response. Other type of stem cells that are being used for retinal diseases trials are stem/progenitor cells derived of fetal central nervous system, including progenitor retinal cells that can have limited self-renewal and tissue-specific differentiation, thus building cells fated only for the retina [103]. In addition, adult stem/progenitor cells are non-pluripotent postnatal cells that can differentiate into some, or all, of the cell types building the organ from which they originate; bone marrow-derived cells, umbilical-derived cells, and mesenchymal stem cells, among others have been employed for treating a variety of retinal disorders [104,105]. Currently, stem cell therapy trials are being conducted for a number of retinal disorders including Stargardt disease (NCT02445612, NCT01469832, NCT03011541, NCT01345006, NCT02941991, NCT02749734), RP (NCT03011541, NCT01068561, NCT01560715, NCT01531348, NCT02280135, NCT03073733, NCT02709876), and Best vitelliform macular dystrophy (NCT02162953), among others. Although cell-based treatments for inherited retinal disease are in their beginnings, they constitute a promising alternative for developing an efficient, mutation-independent, therapy to restore retinal function.

6. Expert commentary

RDs are one of the most genetically heterogeneous human diseases. Collectively, they are the most common cause of inherited blindness and due to its monogenic nature possess an elevated risk of recurrence in pedigrees. Clinical onset, phenotypic presentation, and rate of progression of visual deficit are highly heterogeneous, even in individuals suffering from the same RD subtype, which precludes accurate diagnosis based on retinal phenotype, degree of visual impairment, or other visual functional tests. During the past 15 years, tremendous advances have been achieved in the recognition of the molecular defects underlying dozens of RDs. This knowledge has allowed not only a better genotypic–phenotypic correlation in affected patients, but also has led to an improved comprehension of the molecular pathways involved in normal retinal physiology. Up to now, mutations in more than 250 genes have been identified as responsible of a myriad of retinal dystrophic phenotypes. In addition, it has become evident that a number of genes that were initially associated with syndromic forms of the disease could also result in isolated RD phenotypes when mutated, greatly complicating genetic counseling. Molecular diagnosis in RDs (i.e. the identification of the causal mutation in a given patient) is of extreme importance as it provides a definitive diagnosis, offers a more realistic prognosis information, provides concise information for reproductive planning, and, importantly, is a prerequisite for the enrollment of patients in a number of gene therapy protocols around the world. Thus, obtaining a molecular diagnosis should

be an imperative in the medical approach of individuals suffering from RDs. Since the discovery of the first RD gene in 1990, molecular diagnosis has relied in standard methodologies as PCR amplification and Sanger sequencing in a gene-by-gene basis. Given the extreme heterogeneity of RDs this approach is impractical and expensive, especially in those forms of disease that exhibit variations in clinical presentation or in phenotypes that can be associated to mutations in numerous genes. Next generation sequencing technologies (NGS) has come to revolutionize molecular diagnosis in RDs, allowing the simultaneous characterization of dozens of RD genes (gene panels), the entire coding-sequence of human genes (whole exome) or the entire genome of an individual (whole genome). These approaches have greatly improved the successful molecular diagnostic rate in RD subjects, reaching figures of up to 70%. Although highly efficient, NGS technologies have specific indications depending on criteria as homogeneity/heterogeneity of the observed phenotype, number of loci involved in a particular RD subtype (genetic heterogeneity), or even ethnic-specific mutational particularities. With an increasing number of clinical and preclinical gene therapy protocols for treatment of RDs, the characterization of the underlying molecular defect is nowadays an indispensable element in the care of patients suffering from these visually devastating conditions.

7. Five-year view

In the next five years, the molecular features of novel forms of RDs will be elucidated by means of technologies of massive DNA sequencing. It will be also recognized that numerous RD cases with no mutation in known genes arises from pathogenic variations in areas of the genome that are not generally screened during molecular diagnosis, as intronic or regulatory regions or by gene structural variants. The development of ethnic-based RD gene panels will greatly facilitate the identification of the causal defect by analyzing only genes that have been demonstrated to be frequently mutated in a given population. Molecular diagnosis will be considered an essential tool for clinical follow-up of RD patients with confirmed mutations in genes associated with syndromic forms of the disease, in order to prevent or ameliorate complications. Significant increase in the availability of molecular testing facilities will allow the recognition of the molecular cause in a growing number of RD patients, with the consequent improvement of our knowledge about genotype-phenotype correlations, the discovery of novel disease-associated genes, and the better characterization of the mutational spectrum in patients from different geographic regions. The results of several ongoing gene therapy clinical trials for diverse RD forms will be published, allowing objective evaluation of their efficacy in visual restoration. Finally, extended molecular diagnosis in RDs would lead to an increase in potential use of preimplantation genetic diagnosis for at-risk couples to avoid the birth of children with severe RD forms.

Key issues

- Retinal dystrophies (RDs) are a clinically and genetically heterogeneous group of monogenic disorders arising from progressive dysfunction of photoreceptors or other retinal cell types.
- Collectively, RDs are the most common cause of inherited blindness around the world.
- Extensive genetic heterogeneity is observed in RDs, with over 250 associated genes identified to date.
- Recognition of the causal mutation in a given RD patient is essential for diagnosis confirmation and for provision of clinical prognostic information.
- Benefits of molecular diagnosis also include the delivery of information for reproductive planning in families and the possibility of early enrolling patients in gene therapy trials according to their genotype.
- Novel DNA sequencing technologies are revolutionizing molecular diagnosis of RDs, by allowing the simultaneous screening of hundreds of genes or even complete genomes and substantially increasing the rate of successful genetic diagnosis.
- Clinicians and others involved in the care of RD patients need to recognize the benefits arising from molecular diagnosis and also the advantages and limitations of the novel genetic screening approaches.
- The most successful example of RD gene therapy is the gene replacement for *RPE65*, in Leber congenital amaurosis 2 (LCA2), an early onset form of RD caused by mutations in the *RPE65* gene.
- As a growing number of preclinical and clinical gene therapy protocols for RDs are being conducted, molecular diagnosis is an imperative for patients with these diseases.

Funding

The authors were supported by grants 169352 and 234413 from the National Council for Science and Technology (CONACYT).

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

References

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA. 1977;74(12):5463–5467.
2. Bakker E. Is the DNA sequence the gold standard in genetic testing? Quality of molecular genetic tests assessed. Clin Chem. 2006;52(4):557–558.
3. McClellan J, King MC. Genetic heterogeneity in human disease. Cell. 2010;141(2):210–217.
4. Yu H, Zhang VW. Precision medicine for continuing phenotype expansion of human genetic diseases. Biomed Res Int. 2015;2015:1–4.
5. Koenekoop RK, Lopez I, den Hollander AI, et al. Genetic testing for retinal dystrophies and dysfunctions: benefits, dilemmas and solutions. Clin Exp Ophthalmol. 2007;35(5):473–485.

• **A comprehensive overview about the advantages and limitations of molecular diagnosis in patients with inherited retinal disease.**

6. Korf BR, Rehm HL. New approaches to molecular diagnosis. JAMA. 2013;309(14):1511–1521.
7. Tsang SH, Gouras P. Molecular physiology and pathology of the retina. In: Duane TD, Tasman W, Jaeger AE, editors. Duane's clinical ophthalmology. Philadelphia (PA): Lippincott-Raven; 1996.
8. Daiger SP, Sullivan LS, Bowne SJ. Genes and mutations causing retinitis pigmentosa. Clin Genet. 2013;84(2):132–141.
•• **A comprehensive review summarizing the mutational spectrum in retinitis pigmentosa as well as recent development in the approaches to gene discovery and mutation detection for this disorder.**
9. Sahel JA, Marazova K, Audo I. Clinical characteristics and current therapies for inherited retinal degenerations. Cold Spring Harb Perspect Med. 2014;5(2):a017111.
10. Nash BM, Wright DC, Grigg JR, et al. Retinal dystrophies, genomic applications in diagnosis and prospects for therapy. Transl Pediatr. 2015;4(2):139–163.
11. Broadgate S, Yu J, Downes SM, et al. Unravelling the genetics of inherited retinal dystrophies: past, present and future. Prog Retin Eye Res. 2017;59:53–96.
12. Roosing S, Thiadens AA, Hoyng CB, et al. Causes and consequences of inherited cone disorders. Prog Retin Eye Res. 2014;42:1–26.
13. Aboshiha J, Dubis AM, Carroll J, et al. The cone dysfunction syndromes. Br J Ophthalmol. 2016;100(1):115–121.
14. Zeitz C, Robson AG, Audo I. Congenital stationary night blindness: an analysis and update of genotype-phenotype correlations and pathogenic mechanisms. Prog Retin Eye Res. 2015;45:58–110.
15. Moradi P, Moore AT. Molecular genetics of infantile-onset retinal dystrophies. Eye (Lond). 2007;21(10):1344–1351.
16. Berger W, Kloeckener-Gruissem B, Neidhardt J. The molecular basis of human retinal and vitreoretinal diseases. Prog Retin Eye Res. 2010;29(5):335–375.
17. Dryja TP, McGee TL, Reichel E, et al. A point mutation of the rhodopsin gene in one form of retinitis pigmentosa. Nature. 1990;343(6256):364–366.
18. Rivolta C, Sharon D, DeAngelis MM, et al. Retinitis pigmentosa and allied diseases: numerous diseases, genes, and inheritance patterns. Hum Mol Genet. 2002;11(10):1219–1227.
19. den Hollander AI, Black A, Bennett J, et al. Lighting a candle in the dark: advances in genetics and gene therapy of recessive retinal dystrophies. J Clin Invest. 2010;120(9):3042–3053.
20. Wright AF, Chakarova CF, Abd El-Aziz MM, et al. Photoreceptor degeneration: genetic and mechanistic dissection of a complex trait. Nat Rev Genet. 2010;11(4):273–284.
21. Lee K, Garg S. Navigating the current landscape of clinical genetic testing for inherited retinal dystrophies. Genet Med. 2015;17(4):245–252.
22. Hafler BP. Clinical progress in inherited retinal degenerations: gene therapy clinical trials and advances in genetic sequencing. Retina. 2017;37(3):417–423.
23. Hull S, Arno G, Plagnol V, et al. The phenotypic variability of retinal dystrophies associated with mutations in CRX, with report of a novel macular dystrophy phenotype. Invest Ophthalmol Vis Sci. 2014;55(10):6934–6944.
24. Gregory-Evans K, Kelsell RE, Gregory-Evans CY, et al. Autosomal dominant cone-rod retinal dystrophy (CORD6) from heterozygous mutation of GUCY2D, which encodes retinal guanylate cyclase. Ophthalmology. 2000;107(1):55–61.
25. Ugur Iseri SA, Durlu YK, Tolun A. A novel recessive GUCY2D mutation causing cone-rod dystrophy and not Leber's congenital amaurosis. Eur J Hum Genet. 2010;18(10):1121–1126.
26. Vithana EN, Abu-Safieh L, Allen MJ, et al. A human homolog of yeast pre-mRNA splicing gene, PRP31, underlies autosomal dominant retinitis pigmentosa on chromosome 19q13.4 (RP11). Mol Cell. 2001;8(2):375–381.
27. Rivolta C, McGee TL, Rio Frio T, et al. Variation in retinitis pigmentosa-11 (PRPF31 or RP11) gene expression between symptomatic and asymptomatic patients with dominant RP11 mutations. Hum Mutat. 2006;27(7):644–653.
28. Venturini G, Rose AM, Shah AZ, et al. CNOT3 is a modifier of PRPF31 mutations in retinitis pigmentosa with incomplete penetrance. PLoS Genet. 2012;8(11):e1003040.
•• **This study identified *CNOT3* gene as the main modifier gene determining penetrance of *PRPF31* mutations, via a mechanism of transcriptional repression.**
29. Ganesh A, Keep RB. Genetic testing in retinal dystrophies. Oman J Ophthalmol. 2011;4(3):105–107.
30. Chiang JP, Trzupek K. The current status of molecular diagnosis of inherited retinal dystrophies. Curr Opin Ophthalmol. 2015;26(5):346–351.
31. Fahim AT, Daiger SP, Weleber RG. Nonsyndromic retinitis pigmentosa overview. In: Pagon RA, Adam MP, Ardinger HH, et al., editors. GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993–2017;2000 Aug 4 [updated 2017 Jan 19].
32. Katsanis SH, Katsanis N. Molecular genetic testing and the future of clinical genomics. Nat Rev Genet. 2013;14(6):415–426.
33. Strande NT, Berg JS. Defining the clinical value of a genomic diagnosis in the era of next-generation sequencing. Annu Rev Genomics Hum Genet. 2016;17:303–332.
34. Chiang JP, Lamey T, McLaren T, et al. Progress and prospects of next-generation sequencing testing for inherited retinal dystrophy. Expert Rev Mol Diagn. 2015;15(10):1269–1275.
• **This review compared NGS with other molecular diagnostic strategies for RD patients, emphasizing clinical as well as diagnosis methodologic challenges.**
35. Bernardis I, Chiesi L, Tenedini E, et al. Unravelling the complexity of inherited retinal dystrophies molecular testing: added value of targeted next-generation sequencing. Biomed Res Int. 2016;2016:1–14.
36. Carss KJ, Arno G, Erwood M, et al. Comprehensive rare variant analysis via whole-genome sequencing to determine the molecular pathology of inherited retinal disease. Am J Hum Genet. 2017;100(1):75–90.
•• **This is the largest study published to date employing whole Genome Sequencing for molecular diagnosis of inherited retinal disorders. Advantages of this approach for detecting uncommon pathogenic variants are demonstrated.**
37. Consugar MB, Navarro-Gomez D, Place EM, et al. Panel-based genetic diagnostic testing for inherited eye diseases is highly accurate and reproducible, and more sensitive for variant detection, than exome sequencing. Genet Med. 2015;17(4):253–261.
38. Hehir-Kwa JY, Claustres M, Hastings RJ, et al. Towards a European consensus for reporting incidental findings during clinical NGS testing. Eur J Hum Genet. 2015;23(12):1601–1616.
39. Adam MP, Ardinger HH, Pagon RA, et al. editors. GeneReviews® [Internet]. Seattle (WA): University of Washington; Seattle; 1993–2017. Educational Materials: comprehensive Genome Sequencing and Multi-Gene Panels;2017 Mar 14.
40. Ellingford JM, Barton S, Bhaskar S, et al. Whole genome sequencing increases molecular diagnostic yield compared with current diagnostic testing for inherited retinal disease. Ophthalmology. 2016;123(5):1143–1150.
• **This study showed that WGS methods can detect RD-causing genetic variants missed by other NGS diagnostic methodologies as sequencing of gene panels.**
41. Audo I, Bujakowska KM, Léveillard T, et al. Development and application of a next-generation-sequencing (NGS) approach to detect known and novel gene defects underlying retinal diseases. Orphanet J Rare Dis. 2012;7(1):8.
42. Glöckle N, Kohl S, Mohr J, et al. Panel-based next generation sequencing as a reliable and efficient technique to detect mutations in unselected patients with retinal dystrophies. Eur J Hum Genet. 2014;22(1):99–104.
43. Wang H, Wang X, Zou X, et al. Comprehensive molecular diagnosis of a large chinese leber congenital amaurosis cohort. Invest Ophthalmol Vis Sci. 2015;56(6):3642–3655.

44. Weisschuh N, Mayer AK, Strom TM, et al. Mutation detection in patients with retinal dystrophies using targeted next generation sequencing. PLoS One. 2016;11(1):e0145951.
45. Zernant J, Xie YA, Ayuso C, et al. Analysis of the ABCA4 genomic locus in Stargardt disease. Hum Mol Genet. 2014;23(25):6797–6806.
46. Tanna P, Strauss RW, Fujinami K, et al. Stargardt disease: clinical features, molecular genetics, animal models and therapeutic options. Br J Ophthalmol. 2017;101(1):25–30.
47. Samorodnitsky E, Datta J, Jewell BM, et al. Comparison of custom capture for targeted next-generation DNA sequencing. J Mol Diagn. 2015;17(1):64–75.
48. García-García G, Baux D, Faugère V, et al. Assessment of the latest NGS enrichment capture methods in clinical context. Sci Rep. 2016;6(1):20948.
49. Clark MJ, Chen R, Lam HY, et al. Performance comparison of exome DNA sequencing technologies. Nat Biotechnol. 2011;29(10):908–914.
50. Choi M, Scholl UI, Ji W, et al. Genetic diagnosis by whole exome capture and massively parallel DNA sequencing. Proc Natl Acad Sci USA. 2009;106(45):19096–19101.
51. de Castro-Miró M, Tonda R, Escudero-Ferruz P, et al. Novel candidate genes and a wide spectrum of structural and point mutations responsible for inherited retinal dystrophies revealed by exome sequencing. PLoS One. 2016;11(12):e0168966.
52. Small KW, DeLuca AP, Whitmore SS, et al. North carolina macular dystrophy is caused by dysregulation of the retinal transcription factor PRDM13. Ophthalmology. 2016;123(1):9–18.
53. Aleman A, Garcia-Garcia F, Medina I, et al. A web tool for the design and management of panels of genes for targeted enrichment and massive sequencing for clinical applications. Nucleic Acids Res. 2014;42(W1):83–87.
54. Haer-Wigman L, van Zelst-Stams WA, Pfundt R, et al. Diagnostic exome sequencing in 266 Dutch patients with visual impairment. Eur J Hum Genet. 2017;25(5):591–599.
55. de Goede C, Yue WW, Yan G, et al. Role of reverse phenotyping in interpretation of next generation sequencing data and a review of INPP5E related disorders. Eur J Paediatr Neurol. 2016;20(2):286–295.
56. Vincent AL, Abeysekera N, van Bysterveldt KA, et al. Next-generation sequencing targeted disease panel in rod-cone retinal dystrophies in Māori and Polynesian reveals novel changes and a common founder mutation. Clin Exp Ophthalmol. 2017 May 9 [Epub ahead of print]. DOI:10.1111/ceo.12983
57. Sikkema-Raddatz B, Johansson LF, de Boer EN, et al. Targeted next-generation sequencing can replace Sanger sequencing in clinical diagnostics. Hum Mutat. 2013;34(7):1035–1042.
58. McCourt CM, McArt DG, Mills K, et al. Validation of next generation sequencing technologies in comparison to current diagnostic gold standards for BRAF, EGFR and KRAS mutational analysis. PLoS One. 2013;8(7):e69604.
59. Baudhuin LM, Lagerstedt SA, Klee EW, et al. Confirming variants in next-generation sequencing panel testing by sanger sequencing. J Mol Diagn. 2015;17(4):456–461.
60. Strom SP, Lee H, Das K, et al. Assessing the necessity of confirmatory testing for exome-sequencing results in a clinical molecular diagnostic laboratory. Genet Med. 2014;16(7):510–515.
61. Beck TF, Mullikin JC, NISC Comparative Sequencing Program; Biesecker LG. Systematic evaluation of sanger validation of next-generation sequencing variants. Clin Chem. 2016;62(4):647–654.
- **This study confirms the fidelity and reliability of NGS data comparing it to Sanger Sequencing and supports the notion that NGS data is highly reliable on its own and, in most cases, should not be validated by Sanger Sequencing.**
62. Bravo-Gil N, Méndez-Vidal C, Romero-Pérez L, et al. Improving the management of inherited retinal dystrophies by targeted sequencing of a population-specific gene panel. Sci Rep. 2016;23910.
63. Wang H, Nettleton D, Ying K. Copy number variation detection using next generation sequencing read counts. BMC Bioinformatics. 2014;15(1):109.
64. Ruddle JB, Ebenezer ND, Kearns LS, et al. RPGR ORF15 genotype and clinical variability of retinal degeneration in an Australian population. Br J Ophthalmol. 2009;93(9):1151–1154.
65. Lelieveld SH, Spielmann M, Mundlos S, et al. Comparison of exome and genome sequencing technologies for the complete capture of protein-coding regions. Hum Mutat. 2015;36(8):815–822.
66. Lazaridis KN, Schahl KA, Cousin MA, et al. Outcome of whole exome sequencing for diagnostic odyssey cases of an individualized medicine clinic: the Mayo clinic experience. Mayo Clin Proc. 2016;91(3):297–307.
67. Eden M, Payne K, Combs RM, et al. Valuing the benefits of genetic testing for retinitis pigmentosa: a pilot application of the contingent valuation method. Br J Ophthalmol. 2013;97(8):1051–1056.
68. Hamel C. Retinitis pigmentosa. Orphanet J Rare Dis. 2006;1:40.
69. Werdich XQ, Place EM, Pierce EA. Systemic diseases associated with retinal dystrophies. Semin Ophthalmol. 2014;29(5–6):319–328.
70. Sadagopan KA. Practical approach to syndromic pediatric retinal dystrophies. Curr Opin Ophthalmol. 2017;28(5):416–429.
- **This comprehensive review summarizes the clinical features of systemic disorders that are associated with pediatric retinal dystrophy and highlights the role of genetic testing.**
71. Mole SE, Cotman SL. Genetics of the neuronal ceroid lipofuscinoses (Batten disease). Biochim Biophys Acta. 2015;1852(10 Pt B):2237–2241.
72. Wang F, Wang H, Tuan HF, et al. Next generation sequencing-based molecular diagnosis of retinitis pigmentosa: identification of a novel genotype-phenotype correlation and clinical refinements. Hum Genet. 2014;133(3):331–345.
- **This study contains a molecular analysis of 123 retinitis pigmentosa probands from a wide variety of ethnic backgrounds, including 113 unrelated simplex and 10 autosomal recessive RP (arRP) cases. Molecular characterization of this cohort allowed refinement of clinical diagnoses in a proportion of patients and *CLN3* as a novel non-syndromic retinal disease gene.**
73. Fiskerstrand T, H'mida-Ben Brahim D, Johansson S, et al. Mutations in ABHD12 cause the neurodegenerative disease PHARC: an inborn error of endocannabinoid metabolism. Am J Hum Genet. 2010;87(3):410–417.
74. Nishiguchi KM, Avila-Fernandez A, van Huet RA, et al. Exome sequencing extends the phenotypic spectrum for ABHD12 mutations: from syndromic to nonsyndromic retinal degeneration. Ophthalmology. 2014;121(8):1620–1627.
75. Schaefer E, Stoetzel C, Scheidecker S, et al. Identification of a novel mutation confirms the implication of IFT172 (BBS20) in Bardet-Biedl syndrome. J Hum Genet. 2016;61(5):447–450.
76. Bujakowska KM, Zhang Q, Siemiatkowska AM, et al. Mutations in IFT172 cause isolated retinal degeneration and Bardet-Biedl syndrome. Hum Mol Genet. 2015;24(1):230–242.
77. Petit L, Khanna H, Punzo C. Advances in gene therapy for diseases of the eye. Hum Gene Ther. 2016 Aug;27(8):563–579.
78. Chacon-Camacho OF, Zenteno JC. Review and update on the molecular basis of Leber congenital amaurosis. World J Clin Cases. 2015;3(2):112–124.
79. Sengillo JD, Justus S, Tsai YT, et al. Gene and cell-based therapies for inherited retinal disorders: an update. Am J Med Genet C Semin Med Genet. 2016;172(4):349–366.
80. Chung DC, Traboulsi EI. Leber congenital amaurosis: clinical correlations with genotypes, gene therapy trials update, and future directions. J AAPOS. 2009;13(6):587–592.
81. Soens ZT, Li Y, Zhao L, et al. Hypomorphic mutations identified in the candidate Leber congenital amaurosis gene CLUAP1. Genet Med. 2016;18(10):1044–1051.
82. Bainbridge JW, Smith AJ, Barker SS, et al. Effect of gene therapy on visual function in Leber's congenital amaurosis. N Engl J Med. 2008;358(21):2231–2239.
83. Maguire AM, Simonelli F, Pierce EA, et al. Safety and efficacy of gene transfer for Leber's congenital amaurosis. N Engl J Med. 2008;358(21):2240–2248.
84. Hauswirth WW, Aleman TS, Kaushal S, et al. Treatment of Leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial. Hum Gene Ther. 2008;19(10):979–990.

85. Maguire AM, High KA, Auricchio A, et al. Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial. Lancet. 2009;374(9701):1597–1605.
86. Bainbridge JW, Mehat MS, Sundaram V, et al. Long-term effect of gene therapy on Leber's congenital amaurosis. N Engl J Med. 2015;372(20):1887–1897.
87. Bennett J, Wellman J, Marshall KA, et al. Safety and durability of effect of contralateral-eye administration of AAV2 gene therapy in patients with childhood-onset blindness caused by RPE65 mutations: a follow-on phase 1 trial. Lancet. 2016;388 (10045):661–672.
•• **This study determined the safety of administration of AAV2-hRPE65v2 to the contralateral eye in Leber Congenital Amaurosis patients enrolled in the phase 1 study.**
88. Schimmer J, Breazzano S. Investor outlook: focus on upcoming LCA2 genetherapy phase III results. Hum Gene Ther Clin Dev. 2015;26(3):144–149.
89. Schimmer J, Breazzano S. Investor outlook: significance of the positive LCA2 gene therapy phase III results. Hum Gene Ther Clin Dev. 2015;26(4):208–210.
90. Coussa RG, Traboulsi EI. Choroideremia: a review of general findings and pathogenesis. Ophthalmic Genet. 2012;33(2):57–65.
91. MacLaren RE, Groppe M, Barnard AR, et al. Retinal gene therapy in patients with choroideremia: initial findings from a phase 1/2 clinical trial. Lancet. 2014;383(9923):1129–1137.
92. Edwards TL, Jolly JK, Groppe M, et al. Visual acuity after retinal gene therapy for choroideremia. N Engl J Med. 2016;374(20):1996–1998.
93. Auricchio A, Trapani I, Allikmets R. Gene therapy of ABCA4-associated diseases. Cold Spring Harb Perspect Med. 2015;5(5):a017301.
94. Gal A, Li Y, Thompson DA, et al. Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa. Nat Genet. 2000;26(3):270–271.
95. Vollrath D, Feng W, Duncan JL, et al. Correction of the retinal dystrophy phenotype of the RCS rat by viral gene transfer of Mertk. Proc Natl Acad Sci USA. 2001;98(22):12584–12589.
96. LaVail MM, Yasumura D, Matthes MT, et al. Gene therapy for MERTK-associated retinal degenerations. Adv Exp Med Biol. 2016;854:487–493.
97. Ghazi NG, Abboud EB, Nowilaty SR, et al. Treatment of retinitis pigmentosa due to MERTK mutations by ocular subretinal injection of adeno-associated virus gene vector: results of a phase I trial. Hum Genet. 2016;135(3):327–343.
98. Thompson JA, Itskovitz-Eldor J, Shapiro SS, et al. Embryonic stem cell lines derived from human blastocyst. Science. 1998;282:1145–1147.
99. Schwartz SD, Hubschman JP, Heilwell G, et al. Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet. 2012;379:713–720.
100. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science. 2007;318:1917–1920.
101. Lamba DA, Karl MO, Ware CB, et al. Efficient generation of retinal progenitor cells from human embryonic stem cells. Proc Natl Acad Sci USA. 2006;103(34):12769–12774.
102. Lamba DA, McUsic A, Hirato RK, et al. Generation, purification, and transplantation of photoreceptors derived from human induced pluripotential. PLoS ONE. 2010;85(5):e8763.
103. Yang P, Seiler MJ, Seiler MJ, et al. In vitro isolation and expansion of human retinal progenitor cells. Exp Neurol. 2002;177(1):326–331.
104. Park SS, Bauer G, Abedi M, et al. Intravitreal autologous bone marrow CD34+ cell therapy for ischemic and degenerative retinal disorders: preliminary phase 1 clinical trial findings. Invest Ophthalmol Vis Sci. 2014;56(1):81–89.
105. Rao RC, Dedania VS, Johnson MW. Stem cells for retinal disease: a perspective on the promise and perils. Am J Ophthalmol. 2017;179:32–38.