Essentials in Ophthalmology
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Gyan Prakash
Takeshi Iwata *Editors*

Advances in Vision Research, Volume II

Genetic Eye Research in Asia and the Pacific

Springer

Essentials in Ophthalmology

Series editor
Arun D. Singh

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Gyan Prakash • Takeshi Iwata
Editors

Advances in Vision Research, Volume II

Genetic Eye Research in Asia and the Pacific

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Editors
Gyan Prakash
National Eye Institute
National Institutes of Health
Bethesda, MD, USA

Takeshi Iwata
National Institute of Sensory Organs
Tokyo Medical Center
National Hospital Organization
Tokyo, Japan

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This book – Volume 2 is dedicated to the blind children of the world and the next generation of researchers who are choosing the field of vision research as their career to help understand the biology of eye diseases

Foreword by Arun D. Singh

The Asian Eye Genetics Consortium (AEGC) was established in 2014 to encourage and focus on eye disease research in Asia. With major population distribution and growth expected to occur in Asia, it is another way to approach public health aspects of ophthalmic diseases. As infectious, nutritional, and other preventable causes of vision loss and blindness are gradually overcome, less common entities such as genetic eye diseases become relevant.

With advancements in DNA sequencing, dissemination of technology, and ease of data sharing, genetic eye diseases lend themselves to exploration. AEGC focuses on studies of heretofore neglected Asian populations. The consortium under the able leadership of Gyan Prakash and Takeshi Iwata has brought together contributors from around the world.

The present monograph (second of the series) represents collective work of researchers from the Middle East, South East, and as far as New Zealand including the developed and the developing nations. The topics covered range from exfoliation syndrome, myopia, keratoconus, retinal dystrophies, and retinoblastoma providing unique Asian perspective and challenges.

It is my sincere hope that readers will find as much pleasure reading this volume as the editors and authors had in writing and editing it. If you find "Genetic Eye Research in Asia and the Pacific" informative, it is because (paraphrasing Isaac Newton) "we have seen further, by standing on the shoulders of the giants."

Cleveland Clinic Foundation
Cleveland, OH, USA

Arun D. Singh

Foreword by Peter Wiedemann

Asia as the most populated region in the world is consequently the most affected with regard to eye diseases. For a long time, research has shown that genetic variations are widely involved in ocular diseases. The lack of information on the genetic basis of eye disease in the Asian population is pertinent, and the Asian Eye Genetics Consortium (AEGC) was established in 2014 to close this gap. Focusing and concentrating on research and patient care on eye disease in Asia, this institution has now grown with a worldwide membership and numerous research collaborations. In a first book published in this series "Advances in Vision Research Volume 1, Genetic Research in Asia and the Pacific," the editors Dr. Gyan Prakash from NIH, NEI, USA and Dr. Takeshi Iwata from Japan tried to coordinate the findings from existing research studies on eye disease in Asians, translate them into patient care, and to identify areas for further research.

The doubling time for information in medicine is two and a half years. We benefit from the rapid evolution of basic science in all fields related to biology and medicine, and especially in relation to ophthalmology and vision. The radical advance in the understanding of inherited eye disease has placed a major responsibility on ophthalmologists in their future care of patients. Genetic research findings must be translated to impact and improve patient care at the community level.

Now, only one year after the first volume, the same editors completed this second volume of AEGC Advances in Vision Research. This book is again a milestone: An update on the AEGC and its scientific outreach is given in the first part. The challenges and opportunities for genetic research in Asia and Pacific are then described. Presenting a cross section of genetics research from different Asian countries the editors have collated a masterful and actual review of current knowledge and future demands. Key eye diseases are covered: retinal degenerations, retinoblastoma, glaucoma, myopia, and keratoconus. Assimilating and presenting this wealth of discovery the editors are to be congratulated for their focus on clinically relevant information. This makes the book a major resource for researchers and clinicians.

Light and vision have always promoted human development; loss of sight is among our most basic fears. Ninety percent of the global burden of eye disease is shouldered by developing countries, many of them in Asia. We believe there is a human right to sight and the editors must be congratulated to start and build the AEGC initiative and draw up this report to enhance eye care to people in this region. While some states of Asia have for a long time

had established world-known research centers, the dispersion of genetic research into many more countries is an encouraging signal. The unconfined exchange of information between research institutions will benefit our patients in the long term. The first volume of this series was dedicated to the blind children of the world, their caregivers for the noble cause, and the vision researchers around the world who are finding the solutions to treat the blindness. By close cooperation of scientists and clinicians we will prevent common forms of blindness in the future and reduce the burden of blindness for the benefit of our children.

Peter Wiedemann

International Council of Ophthalmology (ICO)
San Francisco, CA, USA
Leipzig University Eye Hospital
LeipzigGermany

Foreword by Gullapalli N. Rao

One of the greatest advances in eye research during the past quarter century is in the area of genetics. This has led to greater understanding of the genetic basis of many of the ocular conditions. The exploitation of this knowledge to find ways of preserving eye health, preventing some forms of disease, and modify the course of others are the exciting challenges that are subjects of worldwide research currently. Genetics research has become an integral part of most leading eye institutions in the world, and these have not only contributed to major breakthroughs but also to the growth of researchers through their graduate programs.

In the recent past, the proliferation of interest and the establishment of genetics research in Asian institutions is a welcome development. While some parts of Asia have advanced rapidly, the dissemination of this practice into many more countries is an encouraging sign. Better and more research productivity is seen from these centers including greater number of people completing their doctoral programs. This research capacity building augurs well for the future of research in the continent.

The efforts of Drs. Gyan Prakash and Takeshi Iwata have provided a further boost to the collaborative endeavor among different groups in the region, which will further accelerate the research on this front. This second edition of the Asian Eye Genetics Consortium book, *Advances in Eye Research*, presents a rich profile of the genetics research from different countries of Asia. The progress in many of the countries since the last edition is impressive. This is the result of increasing investment in research in many institutions of the region. Entry of more younger generation scientists into eye research, excellent research infrastructure, and increasing number of peer-reviewed publications in respected journals are all positive signs for a vibrant research culture. This is slowly flowing into the clinical education programs with more clinicians engaged in research, both clinical and basic partnering with basic scientists. Hopefully, this will percolate to medical schools in the region. Translational research can only happen when a critical mass of clinicians get into the research mold. Genetics research is a fertile ground that is conducive for clinicians exposed to the culture of research.

The content of this volume is a rich blend of the manifestation of all the above developments. The spectrum of topics provides an excellent profile of genetics of eye problems in Asia and should be a “must” in all the ophthalmic libraries of Asia.

My congratulations to Drs. Prakash and Iwata for this wonderful effort.

L. V. Prasad Eye Institute
Hyderabad, India

Gullapalli N. Rao

Preface

We have come a long way from the humble beginning of Asian Eye Genetics Consortium (AEGC) in 2014 to a very active research based collaboration in vision sciences. The establishment of AEGC has succeeded in creating several international collaborations to understand the biology of eye diseases, training the next generation of research leaders, and application of scientific knowledge in clinical care and treatment. More than one hundred and seventy AEGC members from over twenty countries are regularly interacting to expand our knowledge of eye diseases. In the past year, the consortium has received numerous inquiries from other regions of the world, including Africa and South America for similar research collaborations. At the fifth founding anniversary of the AEGC at ARVO in Honolulu, HI, USA, on May 1st, 2018, the members present at the meeting unanimously approved the expansion of AEGC to Global Eye Genetics Consortium (GEGC, http://gegc.org) effective immediately. GEGC will stand for "a research based consortium for advancing global vision sciences."

A concerted global effort like GEGC has the potential to accelerate the collaborative genetic eye research in generating useful new scientific data to combat eye diseases. The GEGC is seeking to uncover new scientific opportunities and identify shared priorities to create unique international collaborations in genetic eye research. The GEGC is creating opportunity to help establish partnerships among scientists, governments, companies, and non-government organizations from many countries to support international research programs for eye diseases.

We have the great honor and privilege to bring out the second volume of this work on research related to the eye diseases as we have assembled more than one hundred leading researchers from the field of Human Genetics, Ophthalmology, Molecular Biology, Biochemistry, Sensory Sciences, clinical research, and non-governmental organizations to present the status of the growing field of genetic eye research. Our hope is that the second volume proves to be a major stimulus for all researchers, clinicians, clinical researchers, and allied eye health professionals with interest in eye diseases, and accelerates high quality research in our understanding of eye diseases. We were privileged to work with a group of authors who are recognized leaders in their respective areas and who willingly gave their time to contribute to this volume despite their busy schedules. We are forever in their debt.

This book would not have become a reality without the support, encouragement, and assistance of several peers and distinguished colleagues. Chieko

Watanabe, Toshiro Mikami, and Selvakumar Rajendran from Springer provided the continuous support of the project. Dr. Arun Singh of Cleveland Clinic, the series editor, provided the support for inclusion in his acclaimed series. We are very grateful to the valuable support of the leadership, senior management and many distinguished colleagues of National Eye Institute in the USA and Tokyo Medical Center in Japan. Finally, and most importantly we are truly indebted to our family members including, Dr. Savita Prakash, Dr. Fumino Iwata, Dr. Shivaani Prakash, Gary Prakash, and many relatives and friends for their encouragement and continued support throughout the project. We are indebted to all those mentioned above and several others who willingly helped us in our endeavors to put this volume together.

Bethesda, MD, USA — Gyan Prakash
Tokyo, Japan — Takeshi Iwata

Contents

Asian Eye Genetics Consortium (AEGC): The First 5 Years

1

Gyan Prakash, Takeshi Iwata, Sundaram Natarajan, and Paul N. Baird

Abstract

The Asian Eye Genetics Consortium (AEGC, http://asianeyegenetics.org) was established in 2014 to focus on genetic eye research in Asia, the most populated region of the world where limited data are available on genetic variation in eye diseases. The consortium has brought a collective thinking and ideas from the researchers around the world who have interest in genetic eye research in the Asian region. Over 100 eye researchers from eye institutions and hospitals from more than 20 countries including Australia, Bangladesh, China, India, Indonesia, Israel, Iran, Japan, Malaysia, New Zealand, Pakistan, the Philippines, Saudi Arabia, Singapore, South Korea, Sri Lanka, Taiwan, Thailand, Turkey, the UAE, and the USA are now participating in the collaborative research and discussion. The current AEGC collaborations include interacting and collaborating to develop programs to share, catalogue, and research work to identify the genetic aspects of eye diseases in Asia.

Keywords

AEGC · GEGC · NEI · TMC · NISO · Moorfields · AJRVO · AJFTLE · ARVO · APAO · IERG · Database · EyeGene · PFV

G. Prakash (✉)
National Eye Institute, National Institutes of Health, Bethesda, MD, USA
e-mail: gyan.prakash@nih.gov

T. Iwata
National Institute of Sensory Organs, Tokyo Medical Center, National Hospital Organization, Tokyo, Japan

S. Natarajan
Aditya Jyot Research in Vision and Ophthalmology, Mumbai, India

Aditya Jyot Foundation for Twinkling Little Eyes, Mumbai, India

Aditya Jyot Eye Hospital, Mumbai, India

P. N. Baird
University of Melbourne, Melbourne, Australia

1.1 Origin of AEGC

The Asian Eye Genetics Consortium (AEGC) traces its beginning in December 2012 when Dr. Deborah Carper, Former Deputy Director, National Eye Institute (NEI) at National Institutes of Health (NIH) in the USA, introduced Dr. Gyan Prakash at NEI to Dr. Takeshi Iwata at National Institute of Sensory Organs (NISO) at Tokyo Medical Center (TMC) and helped in developing a research collaboration agreement between the two institutions. Subsequent deliberations between the two organizations led to the develop-

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ment of a research collaboration in April 2014 signed by Dr. Paul Sieving, Director of NEI, and Dr. Yozo Miyake (on behalf of TMC) during the World Ophthalmology Congress in Tokyo. The signing of research collaboration between the two institutions began a series of discussions leading to the establishment of AEGC in May 2014 at ARVO in Orlando, USA, by more than a dozen leading eye researchers from around the world.

1.2 AEGC: Current Roles

The Asian Eye Genetics Consortium (AEGC) was established to focus on eye research in Asia, the most populated region of the world where very little has been explored for genetic eye diseases. The consortium has brought together many scientists and clinicians from around the world interested in collaborative international eye research on the patients of Asian region. A concerted global effort like AEGC has the potential to accelerate the collaborative genetic eye research in generating useful new scientific data to help in our understanding of eye diseases. The AEGC is seeking to uncover new scientific opportunities and identify shared priorities to create unique international collaborations in genetic eye research. The AEGC has created a wide opportunity to establish partnerships among scientists, governments, companies, and nongovernment organizations to support research programs for understanding the biology of eye diseases. The consortium has brought a collective thinking from the researchers around the world who have interest in genetic eye research in the Asian region. The theme is going to be expanded in other regions of the world with a goal of creating Global Eye Genetics Consortium (GEGC) soon.

In the last 3 years, several researcher exchanges have taken place providing and strengthening research collaborations on genetic eye research. The scholar's and visitor's programs combined with laboratory and clinic-based training between the participating institutions are one of the key goals of the AEGC programs. Such programs have helped in establishing new eye genetic laboratories in the Asian region and training the interested researchers and clinicians in eye genetic research. In addition, the data sharing is being planned by constructing a common database for AEGC to pool genotype-phenotype information. The AEGC members are working on obtaining new research support grants and corporate support to conduct whole genome sequence DNA samples from the countries that have limited or no funding now to support or conduct research locally.

Over 150 eye researchers from more than 20 countries have become AEGC members since the inception. The members are currently interacting and collaborating to develop programs to share, catalogue, and work to identify the genetic aspect of eye diseases in the Asian countries. The first AEGC book, *Advances in Vision Research – Genetic Eye Research in Asia and the Pacific – Volume I*, edited by Dr. Gyan Prakash and Dr. Takeshi Iwata, was published in May 2017 by Springer (ISBN: 1612-3212). The volume contained the work of more than 100 scientists from the USA, Australia, Europe, and many Asian countries illustrated in 38 chapters [1].

1.3 AEGC Goals

AEGC has the following goals and plans:

1. Share genetic information in the Asian population to rapidly isolate common disease-associated variants.
2. Establish system for accurate diagnosis and grouping of Asian eye diseases.
3. Establish system for cost-effective genetic analysis.
4. Develop a research-oriented database to collect, diagnose and catalog eye diseases in Asia.

5. Support and foster collaboration among the Asian countries for the advancement of research that will provide genetic information in the Asian population.
6. Collaborate with other international or regional organizations with similar goals.
7. Organize and hold regional congresses and other educational and scientific activities to promote goals of the consortium.

Currently, two countries – Japan and India – have established AEGC country consortia.

Japan Eye Genetics Consortium (JEGC) started in 2011 to identify gene mutations responsible for 37 hereditary retinal diseases including hereditary optic neuropathy and hereditary glaucoma in the Japanese population. JEGC has now expanded to 30 university ophthalmology departments in Japan and collected over 2200 Japanese DNA samples. The new genotype-phenotype database was launched on 2017 for collection on phenotypic information for each gene mutation. Additional details on the JEGC are available in Chap. 11 submitted by Dr. Takeshi Iwata.

1.4 Updates on AEGC Sessions and Meetings

The following sessions and meetings have taken place since the publication of AEGC's first book, *Advances in Vision Research – Volume I*.

1.4.1 AEGC Session at ARVO-Asia, Brisbane, Australia

An AEGC session was held at ARVO-Asia on February 5, 2017, in Brisbane, Australia. Dr. Zi-Bing Jin from China started the session by presentation of sporadic patients with inherited retinal dystrophy in China. Dr. Calvin Pang from China talked about the molecular genetics of polypoidal choroidal vasculopathy and age-related macular degeneration. Dr. Takeshi Iwata from Japan gave the updates on expansion of AEGC, and Dr. Mridul Kumar Sarkar from Bangladesh gave a talk on the genetics of congenital cataract in Bangladesh. Dr. Paul Baird from Australia moderated the session.

1.4.2 AEGC Session at Asia-Pacific Academy of Ophthalmology, Singapore

An AEGC session was held on March 3, 2017, at APAO meeting in Singapore. More than eight speakers from several Asian countries gave recent progress report of their genetic research. Dr. Rajkumar Patil from Singapore talked about the prevalence of ocular genetic disorders in Asia. Dr. Hyeong Gon Yu from South Korea gave an update on genetic characterization of Korean retinitis pigmentosa patients. Dr. Rita Sitorus from Indonesia talked about the NDP polymorphism in ROP. Dr. Paisan Ruamviboonsuk from Thailand introduced the GWAS study for AMD and PCV patients in the Thai populations. Dr. Liza Sharmini Ahmad Tajudin from Malaysia informed that she had been collecting DNA from glaucoma patients in Malaysia and presented her work on the progress. Dr. Subhabrata Chakrabarti from India also presented his genetic interaction of different loci in glaucoma patients. The last speaker, Dr. Periasamy Sundaresan from India, gave the multiplex cytokine analysis in the aqueous humor of patients with primary angle glaucoma. The session was moderated by Drs. Paul Baird from Australia, Sundaram Natarajan from India, and Rajkumar Patil from Singapore.

1.4.3 AEGC Meeting and Poster Presentation at ARVO, Baltimore, USA

The fourth annual AEGC meeting at ARVO was held on May 9, 2017, to update the progress of AEGC activities. Drs. Takeshi Iwata from Japan and Paul Baird from Australia moderated the session. Dr. Iwata provided the recent updates on the AEGC programs around the world. Dr. Fujinami from Japan and Dr. Santa Tumminia and Ms. Kerry Goetz from the USA discussed the desired characteristics of the proposed AEGC database. Dr. Gyan Prakash from the USA provided an update on the AEGC book, volume I, and plans for the proposed volume II. Dr. Paul Baird provided the short- and long-term goals of the AEGC organization and discussed various activities related to researcher exchange, webinars, and other educational programs. Dr. Natarajan from India led the discussion on research funding to support the AEGC programs. The discussion was supplemented by Drs. Iwata, Prakash, and Baird and included discussion on government funding from NIH-NEI, JSPS, Australia, DBT India, and several other sources such as industry (Macrogen, etc.), foundations (FEB, etc.), and others.

1.4.4 ARVO 2018 Poster Presentation

An invited poster presentation at ARVO 2018 in Baltimore drew significant attention and discussion at the ARVO meeting. Several new members signed up and discussed collaborative research ideas with their international counterparts. The poster presentation led to several successful new alliances for research partnerships.

1.4.5 AEGC Meeting at Asia-Pacific Academy of Ophthalmology, Hong Kong, China

An AEGC session was held during the APAO meeting in Hong Kong on February 8, 2018, to discuss the progress of genetic research in the AEGC member countries. A total of 30 people attended the meeting. Dr. Takeshi Iwata from Japan summarized the status of the consortium structure, the research funding opportunities, and the status of genotype-phenotype database. Dr. Graham Holder who recently moved from Moorfields Eye Hospital to National University of Singapore is setting up a diagnostic lab with electroretinogram (ERG) and training of young

ophthalmologists. Dr. Muneeb Faiq from All India Institute of Medical Sciences in India gave a summary of the genetic research in India and surrounding countries. Dr. Yudisianil E. Kamal and Dr. Rita S. Sitorus from University of Indonesia gave a presentation on the genetic polymorphism in hepatocyte growth factor and cMET gene as predisposing factors for myopia. Dr. Bo Lei from Henan Eye Institute in China introduced his work on inherited eye disease service in the most populated province in China. Dr. Govindasamy Kumaramanickavel from Aditya Jyot Foundation for Twinkling Little Eyes in India provided an update on diagnostic and sample collection at the first AEGC genetic lab in Mumbai, India. Dr. Paul Baird from University of Melbourne in Australia summarized the talk and discussed the challenges in coordinating research funding and DNA sequencing projects.

1.4.6 Sessions of Indian Chapter of AEGC

In the last 3 years, various research meetings, local AEGC sessions, and CMEs were conducted in India to develop internationally accepted registry for inherited eye disorders. Such programs have helped in establishing first AEGC eye genetic laboratory in Mumbai and promoting eye genetic research in India.

AEGC meeting was held on November 12, 2014, to decide on the diseases to be addressed through AEGC, to identify labs for genetic testing, and to create formats for clinical data collection and documentation. A data sharing is being planned by constructing a common database for AEGC to pool genotype-phenotype information. The AEGC members are working on new research support grants and corporate support to whole genome sequence DNA samples from the countries that have limited or no funding support to conduct research locally at present. Also, AEGC session was organized at All India Ophthalmology Society (AIOS) annual meetings in 2014 and 2015.

In the last 3 years, Aditya Jyot Foundation for Twinkling Little Eyes (AJFTLE) in Mumbai, India, took a lead in inviting the scientists from around the world to stimulate a dialogue on building an AEGC-dedicated lab in Mumbai. AJFTLE was visited by distinguished researchers, including Prof. Shomi Bhattacharya, Head of Molecular Genetics, Institute of Ophthalmology, University College, London; Prof. Sudha Iyengar, Center for Clinical Investigation, Cleveland, Ohio; Dr. Takeshi Iwata, Division Director, National Institute of Sensory Organs, Japan; Prof. Calvin Pang, Director of the CUHK Ophthalmic Research Center; and Dr. Ram Nagaraj, Professor, University of Colorado Denver, USA.

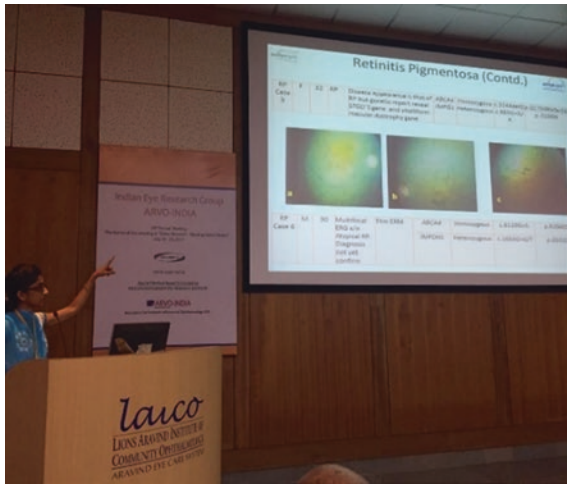
Dr. Takeshi Iwata delivered the first Dr. A. P. J. Abdul Kalam Public Endowment Lecture on "December 14, 2016," and was the recipient of the scroll of honor and plaque in India. Under his guidance, Aditya Jyot Research in Vision and Ophthalmology *(AJRVO)*, R&D unit of AJFTLE, has started research on age-related macular degeneration. The purpose of Dr. Takeshi Iwata's visit to Mumbai, India was to stimulate various research activities related to AEGC through collaboration. AJFTLE (India) and NISO (Japan) have agreed for a collaborative research.

Dr. Iwata visited the AJRVO Kalam Genetic Lab at Thane.

Dr. Iwata receiving 1st Dr. A. P. J. Abdul Public Endowment award

The 24th annual meeting of the Indian Eye Research Group (IERG) – ARVO India Chapter Meeting – was held at Aravind Medical Research Foundation, Madurai, Tamil Nadu, from July 28 to 29, 2017. The AEGC component of AJFTLE research team was represented at IERG 2017.

Prof. Govindasamy Kumaramanickavel, Research Director at AJRVO, was conferred the prestigious D. Balasubramanian Award *by Dr. P. Namperumalsamy, Chairman Emeritus of Aravind Eye Hospital, Madurai*, for his contribution in genetic research. He delivered an oration on "A Journey in Darkness: Pathway to Ophthalmic Genes" at the conference that included the establishment of AEGC chapter in India.



AJFTLE participated in the poster and presentation session at IERG. Dr. S. Rajkumar, Lab head, AJRVO, presented a poster on "Identification of Potential Candidate Genes of Persistent Fetal Vasculature (PFV), A Congenital Ocular Anomaly Using Combinatorial Whole Exome Sequencing and Gene Prioritization Tools." Ms. Siddhita Nare, Junior Research Fellow, AJRVO, presented free paper on "Screening of Genetic Variations in Different Ocular Disorder Using Targeted Exome Sequencing (TES) Panel."

In AIOS 2018 Conference, Aditya Jyot conducted Instructional Course on Ophthalmic Genetics under the leadership of Prof. Govindasamy Kumaramanickavel.

1.5 The First AEGC-Dedicated Lab in India

In the year 2016, the AJFTLE in India established a genetic unit – AJRVO – at Thane, Maharashtra, headed by Prof. S. Natarajan and Prof. Govindasamy Kumaramanickavel. This genetic laboratory, under the AJFTLE, is recognized by the Government of India – Department of Science and Industrial Research – as small industrial research organization. The lab was inaugurated by Dr. Takeshi Iwata.

AJRVO Lab in Thane, India

The major focus of the AEGC – genetic unit of AJFTLE – is to study the genetic and epigenetic mechanisms of diabetic retinopathy, age-related macular degeneration, glaucoma, cataract, myopia, and inherited eye diseases through genomics and proteomics tools. The genetic laboratory is

equipped with all basic amenities and instruments needed to perform in-house genetic analysis.

AJFTLE is in the process of making a biobank for all available clinical specimens obtained through surgical procedures during intervention. The samples including peripheral blood for DNA and RNA isolation, plasma, serum, aqueous, and vitreous for biomarker analysis and lens aspirate, trabecular meshwork, epiretinal membrane and inner limiting membrane for epigenetic, proteomic, and microRNA analysis have been collected and stored at −80 °C.

1.6 AEGC Database

Most of our knowledge that underpins retinal disease has come from studies based in Europe and North America; this is particularly evident in the composition of groups involved in large international consortia. As a result, most of the molecular information that underpins retinal disease and the genes involved in its etiology are derived from these populations. Africa, South America, and Asia are underrepresented in this global knowledge base. However, Asia has a third of the global population, and as such information gained from the study of retinal diseases in this region will greatly advance our understanding of its molecular mechanisms and the pathobiology of eye diseases.

To accomplish the goals of the AEGC, there is a need for a comprehensive database that must allow for all interested groups across the region to be placed on an equal platform with the emphasis being on high-quality data and sharing of this data at a de-identified level. Therefore, the success of the AEGC (and future GEGC) is underpinned by the type of database that can be used to share clinical and genetic data.

There are many unique advantages of having a region-based database. These include:

- Better identification of mutations and genes involved in a retinal disease.
- Identify mutations that are novel and specific to the Asian population.
- Aid in the diagnosis of patients.
- Ability to look at different aspects of disease such as country-level mutations.
- Identify rare disease/subtype/clinical feature and build number of samples.
- Generation of large data sets through more samples.
- Ask a broader range of questions that might already be possible.
- Opportunity to be involved with other groups in the region.
- Apply for funding and other opportunities.
- Write papers with bigger impact.

To facilitate the best design of a database, a series of meetings were held with experts from around the world in the last 3 years to garner experience from different data platforms that were being used. These included meetings with Dr. Takeshi Iwata from Japan providing the example of the Japanese exome database, Dr. Andrew Webster from Moorfields Eye Hospital detailing how data is handled in London, and Ms. Kerry Goetz and Dr. Santa Tumminia at ARVO and at NEI, USA, to assess "EyeGene." While these databases were exceptional and had their own merits, it was clear that the AEGC would need to devise its own database setup. The main considerations to take into account were as follows:

- Who would undertake the data management?
- What kind of database was needed?
- Would the database be at one site or mirrored at several sites and if so, where?
- What kind of data should be collected?
- What data would and could be shared?
- How to ensure reproducibility of the collected data.
- How would harmonization/data cleaning/quality control data be established?
- Who would provide database/data query support to users?

The groups approached around the world all spoke of an aspiration of having databases that "could talk to each other," and this underlying thread will be incorporated into a design for a database.

1.6.1 AEGC Infrastructure

To underpin the database aspirations, it was clear that Internet access and digitalization would be essential to collect data from not only remote or rural sites but also major urban sites. Therefore, establishing country leaders to identify locally interested groups and facilitate the identification of patients in their country would be a powerful resource. Such undertakings of building nodes of local expertise have now begun with the establishment of the AJFTLE at Thane, Maharashtra, India, under the guidance of Drs. S. Natarajan and G. Kumaramanickavel.

1.6.2 Clinical Data

Given that medical clinics are extremely busy and that medical staff are time constrained, there was a consideration as what data should be collected. While data would need to be collected on an individual for diagnosis and verification of the phenotype by an independent expert, it was also essential that the collecting clinician should not be overburdened with multiple fields of data entry that would need to be completed. Critically, it was observed that many of the fields necessary for the database are part of a regular clinical treatment, and so the requirement would be to ensure that images or clinical data were collected at an appropriate image resolution for assessment and that the clinical data collected met international standards for diagnostic characterization of the retinal disease. Therefore, one of the goals of AEGC is to build this level of excellence across the region.

1.6.3 Genetic Data

While clinical data is readily available, the genetic data related to a patient may not be available at the local level. This may come about through lack of resources, expertise, manpower, etc. AEGC aims to facilitate the elucidation of molecular changes that occur in retinal diseases and feed this information back to the treating doctor. Genetic data to be assessed would need to be shipped to a site that could undertake the genetic studies. Through discussions around the region, it was noted that not all countries such as India and China would be able to export tissue overseas for such analysis. To overcome this hurdle, Drs. Iwata and Baird indicated that there could be a two-tier system where countries with the capacity to undertake genomic sequencing would be best placed to perform this in their home country, whereas countries with no such facilities could send these tissues overseas for analysis.

1.6.4 Outcomes

The outcomes of the database work are to:

- Provide a catalogue of mutations in retinal disease across the region.
- Provide a diagnostic molecular tag for a patient to aid in diagnosis.
- Provide information to identify missing genetic information that could explain retinal disease.
- Provide insights into molecular mechanisms of disease.
- Provide a mechanism to map mutations across the region and within countries to look for main mutational effects.
- Provide opportunities to develop therapies for different types of retinal disease.

1.6.5 The Database Today

Initially, it is being planned that the AEGC database will be housed at the Tokyo Medical Center under Dr. Iwata's support. Each group will have access to their own data but not to other groups. The administrator in Japan will have access to the data that will be de-identified. Each group will have a unique identifier for their patient where they will be able to reidentify the patient and hence provide an unequivocal diagnosis back to their patient. No identifying information will be transmitted between sites. Local ethics will be obtained for all material.

1.6.6 Future Work

Initial trials will need to be conducted to ensure that the transport of samples, processing, and transmission of genetic data can be established. Assessment of the mutation will need to be undertaken either in Japan or in a host country with capacity to undertake this analysis. In all cases the genetic data will need to be married to the clinical data.

It is envisaged that once the database is in place, then samples and/or data will begin to be uploaded rapidly. Once the process is established, we will begin to expand this concept to other areas including Africa and South America. The hope is that a unified system to benefit retinal patients can be established aiding in developing new treatments for this group of diseases.

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A Bibliometric Analysis of AEGC Scientific Outreach

2

Pamela C. Sieving

Abstract

The Asian Eye Genetics Consortium (AEGC) encourages and facilitates research and clinical collaborations to understand the genetic etiology and physiology of ophthalmic diseases and work toward clinical care and treatment. Bibliometric analysis of ophthalmic genetic publications in Asian countries provides a tool to better understand research strengths and challenges.

Keywords

Ophthalmology · Eye diseases · Bibliometrics · Asia · Research collaboration · Research funding · Genetics

2.1 Introduction

Bibliometric research, sometimes also termed "scientometrics," analyzes the metadata of publications to identify significant elements of the research reported beyond the scientific content: who is publishing, where is the research done, how is it funded, where is it published, and what are the trends and challenges, the successes, and the gaps. Citation impact is one element of this research, but Journal Impact Factors™ and numbers of citations are not the primary focus.

The earliest attempts to analyze a body of literature include work by Cole and Eales, who looked at the literature of comparative anatomy from 1540 to 1860 [1], noting the first "outcrop of publications" between 1540 and 1575; they identified centers of scholarship such as Padua, fretted over apparent periods of lack of interest or progress in anatomical studies, and invented combined "working years" as a metric: they count 2100 working years, for the active publication lives of prominent anatomists between 1650 and 1700. In 1962, Raisig published a paper on "statistical bibliography in the health sciences," [2] identifying some of the key principles of bibliometric analysis several years before Pritchard first used the term "bibliometrics" [3].

Earlier bibliometric studies in ophthalmology and vision have sometimes attempted to look at the entire field (Guerin [4], Mandal [5], Ohba [6], Schulz [7]) but more typically focused on a specific country, such as Davis, for Australia [8]; Kumaragurupari, for India [9]; Pahor, for Slovenia [10]; Rahman, for Japan [11]; Risal, for Nepal [12]; Wolfram, for Germany [13]; and Yohendran [14], for the indigenous population of Australia, or region, such as Ragghianti [15], for five South American countries [ref], and Sweileh [16], for Arab-focused ophthalmology. Others have analyzed the literature of specific diseases or ophthalmic knowledge: Boudry [17], for eye

P. C. Sieving (✉)
National Eye Institute, National Institutes of Health, Bethesda, MD, USA

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neoplasms; Cardona [18], for contact lens; Dai [19], for traumatic optic neuropathy; Dong [20], for glaucoma; Fan [21], for cataract and corneal refractive surgery; Mimouni [22], who compared pediatric- and adult-focused ophthalmic research; Pekel [23], for corneal transplantation; Royle [24], for macular disease in the United Kingdom; Şenel [25], for Behçet disease; Wang [26], for ocular ischemic syndrome; and Wen [27], for cataract research.

This chapter reports the findings of a study of ophthalmic genetic publications by researchers in Asian countries, using the World Health Organization's designation of countries in the Southeast Asia, the Western Pacific, and the Eastern Mediterranean regions. In addition to the standard bibliometric analyses, the results may help identify researchers with access to unique genetic populations, highlight opportunities for private and public funders to identify possible research facilities while helping researchers identify potential funders, and encourage collaborations beyond those already recognized.

2.2 Methods

The *Web of Science*™ (*WoS*) database (Clarivate Analytics, Philadelphia, PA, USA) was used both to identify research publications authored by researchers from Asian countries and to conduct bibliometric analysis on the publications. *WoS* provides citation-level access to research publications in journals judged to be significant in their subject areas, as indicated by the assignment of Journal Impact Factor™ (JIF) rankings. Citations include author institutional affiliations for all authors; this information is not consistently provided by the more generally available *MEDLINE®/PubMed®* database for most of the publication years studied, hence the choice of *WoS*. *WoS* also consistently includes data for both funding agencies and grant numbers. In addition, *WoS* provides point-and-click analytics for almost every data field which can be captured from the individual article and its bibliographic citation: language of publication, document type, broad subject groupings for journals (corresponding to the JIF categories), and both country and institutional elements of the authors' affiliations. Citation counts, based on reference lists in all journals assigned JIFs, and page views since 2013 identify and quantify one form of impact of journals, articles, authors, institutions, granting agencies, and specific grants.

A search was constructed to identify articles meeting four criteria: ophthalmology and vision-related publications; by at least one author from an Asian country, not limited to AEGC-participating members; with genetic content; and published from 2000 through 2017. While bibliographic indices such as *MEDLINE®* provide subject indexing using a controlled taxonomy, this is not available in *WoS;* instead, lists of terms likely to be used in the titles of target articles were constructed, based on terminology in the *MEDLINE®* MeSH® taxonomy for eyes and eye diseases and for genetics and subject "hedges" developed by the author. WoS automatically invokes lemmatization, expanding the search to include variations in spelling and usage (e.g., "oedema" for "edema," "eyes" as well as "eye"). Two parallel searches were run: the first used a two-part structure of eye and vision terminology + genetic terminology; the second used genetic terminology + the *WoS* classification of journals in the field of ophthalmology. The two searches were combined to eliminate duplicate citations, and the additional element of country-level author affiliations added to the result. Only articles published from 2000 through 2017 were included in the final data set. Review of the data revealed citations from unrelated fields such as astronomy and oceanography in which vocabulary overlapped; those citations were manually removed.

2.3 Results

Number of Publications The final data set for analysis included 24,715 citations: 21,316 articles, 1559 meeting abstracts, 992 reviews, 615 letters, and 464 proceedings papers. 24,636 of the citations were to publications in English (99.684%); other *languages* included Chinese,

Japanese, German, Korean, French, Portuguese, Romanian, Spanish, and Turkish.

Year of Publication Publications increased steadily by year, from 706 in 2000 to 2419 in 2017. As a check on accuracy, a basic search was run in *MEDLINE®/PubMed®* for citations indexed using the most relevant MeSH® taxonomy ("eye diseases/ge" or "eye diseases, hereditary") for 2000 and 2016, the last year for which subject indexing is complete; there were 410 articles in 2000 and 1091 in 2016, a similar rate of increase, providing additional confidence that this dramatic increase in number of publications is genuine.

Country Twenty-one countries are formal members of the AEGC; their contributions ranged from 4655 papers (People's Republic of China) to (Cambodia). One hundred seven countries were represented by at least one author on these papers. The highest number of contributions by authors in non-AEGC countries was those from England (492), Germany (367), the Netherlands (249), Canada (208), France (180), and Pakistan (174). Eighteen countries contributed to only a single paper and 11 to two papers; in total, 44 countries contributed to five or fewer (Tables 2.1, 2.2, and 2.3).

Journals *Web of Science*™ sorts journals into broad categories which are then used to compare journals for which Journal Impact Factors have been computed; the "Ophthalmology" category for 2016 publications includes 59 journals (*Journal Citation Reports*™, 2016). Of the 24,715 papers in this data set, 9812 (39.713%) were published in journals in this category: *Investigative Ophthalmology & Visual Science* (published 2440, 9.876%), *Molecular Vision* (1175, 4.756%), *Experimental Eye Research* (477, 1.931%), *International Journal of Ophthalmology* (373, 1.931%), *British Journal of Ophthalmology* (367, 1.485%), *Current Eye Research* (348, 1.409%), *Clinical and Experimental Ophthalmology* (342, 1.384%), *Japanese Journal of Ophthalmology* (323, 1.307%), *Cornea* (286, 1.158%), and *American Journal of Ophthalmology* (280, 1.133%) (Table 2.4).

Table 2.1 Publications by AEGC member countries, 2000–2017

Australia	1253
Bangladesh	7
PR China	4655
Egypt	15
India	1046
Indonesia	22
Israel	43
Iran	24
Japan	2194
South Korea	960
Malaysia	90
New Zealand	134
Philippines	8
Singapore	365
Saudi Arabia	69
Switzerland	97
Taiwan	402
Thailand	68
Turkey	50
United Arab Emirates	5
United States	1936

Table 2.2 Publications by Non-AEGC Asian countries

Cambodia	2
Iraq	4
Jordan	4
Kuwait	2
Mongolia	3
Myanmar	1
Nepal	15
Pakistan	174
Papua New Guinea	2
Qatar	9
Sri Lanka	6
Vietnam	15

The remaining 14,903 articles were published in 150 non-ophthalmology categories; leading in numbers of ophthalmic genetics published were these categories: biochemistry/molecular biology (3064, 12.401%), genetics/heredity (2002, 8.103%), neurosciences (1395, 5.646%), cell

Table 2.3 Publications by countries collaborating with AEGC-based researchers (top 20 by publications)

England	492
Germany	367
Netherlands	249
Canada	208
France	180
Italy	126
Spain	87
Scotland	80
Sweden	71
Belgium	61
Denmark	60
Brazil	59
Austria	37
Norway	37
Poland	37
Northern Ireland	33
Wales	28
Finland	25
Greece	25
Argentina	24

Table 2.4 Vision journals

IOVS	2440
Mol Vision	1175
Exp Eye Res	477
Int J Ophthalmol	373
BJO	367
Curr Eye Res	348
Clin Exp Ophthalmol	342
Jpn J Ophthalmol	323
Cornea	286
Am J Ophthalmol	280
Graefes Arch Clin Exp Ophthalmol	276
Ophthalmology	246
Ind J Ophthalmol	211
Eye	203
Arch Ophthalmol/JAMA	
Ophthalmology	168
Ophthalmic Res	153
Ophthalmic Genet	142
BMC Ophthalmology	114
Retina	113

Table 2.5 Non-vision journals

PLoS One	713
Sci Rep	297
Biochem Biophys Res Commun	242
J Biol Chem	166
Am J Hum Genet	141
Mol Med Rep	134
Chin Med J	127
Am J Med Genet A	112
Hum Mol Genet	111
J Hum Genet	103
PNAS	100
Int J Clin Exp Pathol	93
J Invest Dermatol	92
Oncotarget	88
Int Med	85
Gene	84
Int J Clin Exp Med	80
Neurosci Lett	77
J Dermatol	74
Am Pathol	72

biology (1288, 5.213%), medicine research, experimental (1279, 5.177%), multidisciplinary (1220, 4.938%), oncology (879, 3.558%), clinical neurology (683, 2.764%), medicine, general internal (620, 2.509%), and endocrinology/metabolism (613, 2.481%) (Table 2.5).

The top 100 journals in number of these papers published include 42 ophthalmology and vision journal; this accords with the c40% of articles published in journals in the Ophthalmology *WoS* subject category. Three of the 42 are open access titles, included in the *Directory of Open Access Journals*, (*BMC Ophthalmology*, *Indian Journal of Ophthalmology*, and *International Journal of Ophthalmology*). *WoS* identifies 9933 of the articles as being available via some form of open access, 40.2%; approximately 24.4% of articles indexed by *MEDLINE*® are identified as being free full text.

Authors 56,541 individual authors are credited on these papers; 500 of them authored at least 34 papers each. In 2017, a total of 12,001 individual authors were credited on the 2419 papers; in 2000, 2882 authors were credited on 706 papers.

Wang Ying (Capital Medical University, Beijing) authored 245 papers; David A. Mackey (University of Western Australia, Perth), 244; Liu Yu (Sun Yat-sen University, Guangzhou), 207;

Jamie E. Craig (Flinders University, Adelaide), 205; Shigeru Kinoshita (Kyoto Prefectural University of Medicine), 204, were the others with more than 200 publications in this set.

Funding Agencies 933 funding agencies were acknowledged in these publications; *WoS* notes, however, that 12,135 (49.116%) of these papers did not contain data for this field. This is likely due to a change in practice by authors and in part due to the increased pressure from funders to see acknowledgments in papers resulting from their funded research. Many funders now also require deposit of the manuscripts or the published papers in a freely accessible archive (such as PubMed Central®, maintained by the US National Institutes of Health and indexed in *PubMed*®) or on the authors' institutional or personal Web site. The reader is referred to ROARMAP for a current compilation of funder policies in this area (http://roarmap.eprints.org). Note: variations in style of authors' attribution of funding necessarily mean some are overlooked, and only those funding at least eight publications are included here; listed here are some of programs most active in supporting this research and the number of papers acknowledging each:

National Natural Science Foundation of China, 2875 (of those funding at least 8).
National Eye Institute, 804.
NIH and individual institutes (including NIDDK, NINDS, NCRR, NIGMS, NHLBI, NICHD, NIDCD, NIAMS, NHGRI, NIA, NIAID, and NIGMS), 1325.
Research to Prevent Blindness, 378.
Ministry of Education, Culture, Sports, Science and Technology of Japan, 954.
National Basic Research Program of China, 501.
Ophthalmic Research Institute of Australia, 130.
Australian Research Council, 285.
Wellcome Trust, 130.
Foundation Fighting Blindness, 74.
Fight for Sight, 59.

Many small funds and charities receive credit: March of Dimes (8), Maurice and Phyllis Paykel Trust (8), Sigrid Juselius Foundation (9), Muscular Dystrophy Association (9), Uehara Foundation (10), and Moorfields Eye Charity (10).

Grants When the information is provided in the acknowledgment sections of publications, *WoS* includes grant numbers in the metadata for each citation, as does *MEDLINE®;* this directly searchable field in the database record allows funders and other researchers to easily identify publications resulting from funded research. A single grant from the National Basic Research Program of China 973 program of the papers in this data set; funding from an internal mechanism at the Chongqing Key Laboratory of Ophthalmology funded 77 papers; one from the Key Project of Natural Science Foundation (China) funded 57; another, from the National Health and Medical Research Council (NHMRC) and Centre for Clinical Research Excellence Grant, Canberra, was acknowledged in 50 papers. An NIH National Eye Institute grant funded 41 papers.

Organizations Nearly 12,000 institutions are identified in the author address information in this data set. The University of Melbourne led with 884 publications, followed by the University of Sydney (781), Sun Yat-sen University/Zhongshan Ophthalmic Center (764), the University of Tokyo (628), the University of California system (566), Shanghai Jiao Tong University (557), Kyoto University (554), the National University of Singapore (538), the University of London (528), the Chinese Academy of Sciences (522), the Centre for Eye Research Australia (508), the University of Western Australia (508), and Osaka University (506). US National Eye Institute researchers authored 266 papers, and researchers at other NIH institutes such as NCI 63 and NHGRI 62 contributed as well, for a total of 491 papers. A sampling of the other organizations represented include Harvard University/Massachusetts Eye and Ear Infirmary 415; LV Prasad Eye Institute 312; Royal Victorian Eye and Ear Hospital,

Table 2.6 Institutional affiliations/publications

University of Melbourne	884
University of Sydney	781
Sun Yat-sen University/Zhongshan Ophthalmic Center	764
University of Tokyo	628
University of California system	566
Shanghai Jiao Tong University	557
Kyoto University	554
National University of Singapore	538
University of London	528
Chinese Academy of Sciences	522
Centre for Eye Research Australia	508
University of Western Australia	508
Osaka University	506
Harvard University/Massachusetts Eye and Ear Infirmary	415
Royal Victorian Eye and Ear Hospital, Melbourne	412
LV Prasad Eye Institute	312
US National Eye Institute	266
Moorfields Eye Hospital, London	181
Aravind Eye Hospital/Aravind Research Foundation, Madurai	173
Hong Kong Polytechnic University	105
King Khaled Eye Specialist Hospital, Riyadh	34
University of Sao Paulo	24

Melbourne, 412; Moorfields Eye Hospital, London, 181; the University of Michigan 277; Aravind Eye Hospital/Aravind Research Foundation, Madurai, 173; the Hong Kong Polytechnic University 105; McGill University, Montreal, 60; the University of Tübingen 53; the Karolinska Institute, Stockholm, 48; the University of Zürich 42; Queen's University Belfast 38; the University of Helsinki 38; King Khaled Eye Specialist Hospital, Riyadh, 34; the University of Paris 62; and the University of Sao Paulo 24. This is a sampling rather than a systematic listing, meant to indicate the geographic and institutional breadth of research efforts in ophthalmic genetics (Table 2.6).

Citations More than 80 of the 2017 publications have already received at least five citations. Of those, only six had three or fewer authors. By contrast, 17 of the most cited articles from 2000 had 3 or fewer authors.

Highly Cited Papers The most cited paper in this data set, by Zuchner et al. [28], was cited more than 800 times at the time of data collection. Notably, the *WoS* record lacks funding information, as many in the early years of the set do; the article itself acknowledges funding from the Deutsche Forschungsgemeinschaft, NIH, the University of Antwerp, and the Belgian Federal Office for Scientific, Technological and Cultural Affairs. Its 11 authors have institutional affiliations, all universities or research institutions, in 7 counties. The second most cited, by Heier et al. [29], had been cited 683 times; it includes 14 authors, from 6 counties, both universities and industries, and cites a total of 22 funding sources. More than 450 of the papers in this data set have been cited at least 100 times. Forty-eight are ranked as "highly cited" by *WoS*.

2.4 Discussion

The results section has provided the reader with both specific and indicative information about the range of publications in the broad area of ophthalmic genetics for the most recent 17 years with at least one author from an Asian country. That researcher may be an established senior clinician, a graduate student, a recently returned fellow, or postdoc who trained in an active lab in another hemisphere.

One use for this data is to compare countries, for example, a developing and a developed country. Looking at India:

Indian authors are identified on 2242 of the 24,715 records for publications (0.9%). 1101 of those papers were published in journals classified as "ophthalmology" by *Web of Science*™, 49.240%, a higher than average choice of ophthalmology and vision journals to disseminate their research. *Citation studies* for this data set include an H-index of 57; average citations per publication is 10.55; the citations were cited by a total of 17,328 other articles (excluding self-citations, 16,410); the publications average 1245 citations per year. *Yearly productivity*: the number of publications grew from 35 in 2000 to 242 in 2016 and 239 in 2017, with a jump from

164 in 2012 to 229 in 2015. *Journals* differed from the pattern for the entire data set: 185 of the papers appeared in the *Indian Journal of Ophthalmology* (8.274%), 143 in *Molecular Vision* (6.395%), 131 in *IOVS* (5.859%), 48 in *Eye* (2.141%), and 47 in *British Journal of Ophthalmology* (2.096%); only 3 of the top 20 journals were not classified as "ophthalmology" titles. Non-vision journals in which Indian authors published included *PLoS One*, 52 articles (2.319%), followed by the *American Journal of Human Genetics*, 19 papers (0.847%), and *Journal of Genetics*, 17 (0.758%). The *Indian Journal of Ophthalmology* was conferred its first Journal Impact Factor™ for the 2010 publication year; this additional quality designation may have persuaded Indian authors to publish in this journal preferentially. Indian authors collaborated with researchers from the following *countries*: the USA (336, 15.027%), England (97, 4.338%), Australia (82, 3.667%), People's Republic of China (70, 3.131%), the Netherlands (49, 2.191%), Japan (36, 1.610%), Singapore (34, 1.521%); and researchers from 18 countries (Canada, France, Italy, Saudi Arabia, Brazil, Belgium, Switzerland, Turkey, South Korea, Spain, Israel, Argentina, Malaysia, Pakistan, Northern Ireland, Hungary, South Africa, and Sweden) collaborated with Indian researchers on at least ten publications. Fifty-five additional countries contributed with 1–9 publications. Sixteen AEGC member countries were among those collaborating on these publications. Approximately 1000 *funding agencies* were credited in these papers. The Indian Department of Biotechnology funded 201 (8.989%), followed by the Indian Council of Scientific and Industrial Research (138) and Indian Council of Medical Research (78). The US National Eye Institute (58) and other National Institutes of Health institutes and centers (56) provided 5.098% of the funding for the research presented in these publications. Private funders included the Hyderabad Eye Research Foundation (49), Research to Prevent Blindness (33), the Foundation Fighting Blindness (16), Alcon (15), the Champalimaud Foundation (15), and the Wellcome Trust (8).

The *most cited paper* including Indian authors is by Fritsche et al. on seven new loci associated with age-related macular degeneration (361 citations). This paper is perhaps typical of high-powered genetic research: 136 authors from a total of 14 countries, funding by major US and British government agencies. The second most cited paper, at 316 cites, is by Bork and 16 additional authors' 2001 paper on Usher syndrome; five countries are represented.

Equivalent data for Japan:

Japanese authors are associated with 7728 papers in this data set, 31.268%. Publications *per year* have varied from 388 (2011) to 492 (2016), not varying more than 10% between successive years. *Citation studies*: this set of 7728 papers has an H-index value of 133, with an average of 19.55 citations to each publication. A total of 98,301 papers have cited the 7728 publications; excluding self-citations, the total is 94,246; 7952 is the average number of citations per year.

Ophthalmic journals in which Japanese ophthalmic researchers choose to publish are similar to those of the entire population in this data set, except that the *Japanese Journal of Ophthalmology* ranks higher: *IOVS* 953 (12.332%), *Japanese Journal of Ophthalmology* 287 (3.714%), *Experimental Eye Research* 178 (2.303%), *Molecular Vision* 175 (2.264), *American Journal of Ophthalmology* 154 (1.993%), and *British Journal of Ophthalmology* 121 (1.566%). Non-ophthalmic journals: used *PLoS One* 162 (2.096%), *Biochemical and Biophysical Research Communications* 108 (1.398%), *Internal Medicine* 83 (1.074%), *Journal of Biological Chemistry* 78 (1.009%), *Scientific Reports* 68 (0.880%), and *Journal of Human Genetics* 59 (0.763%). The *countries of the collaborating authors* are the USA (1162, 15.036%), England (239, 3.093%), People's Republic of China (223, 2.886%), Germany (206, 2.666%), France (133, 1.721%), Canada (102, 1.320%), South Korea (94, 1.216%), Australia (88, 1.139%), the Netherlands (84, 1.087%), and Italy (79, 1.022%). *Funding* was led by the Ministry of Education, Culture, Sports, Science and Technology of Japan, which funded 785 of

these papers, 10.158%; the Japan Society for the Promotion of Science, 237 (3.067%); the US National Eye Institute 211 (2.731%); the US National Institutes of Health other than NEI, 289 (3.734%), a total percentage similar to but one point higher than that for Indian publications; and the Takeda Science Foundation, 130, 1.682%. Other leading funders were Japanese governmental agencies and foundations. The *most cited paper* including Japanese authors is that of Zuchner and the second that of Heier, both already discussed. The most cited paper with a first author at a Japanese institution is that by Yoshida [30]; it may be of interest to consider that this paper was published in the first volume of a new journal; thus the paper was submitted without consideration of the Journal Impact Factor™ associated with the journal, yet it has been cited nearly 500 times.

2.5 Conclusions

Bibliometric analysis of publications focused on a knowledge domain or geographic region provides a "where we have been/where we are" snapshot of scholarly communication and scientific achievement. Findings here confirm that ophthalmic genetics is an increasingly active and productive research front, with a rapidly increasing pool of researchers contributing to knowledge of ophthalmic genetics. The impact of the increasing numbers and variety of funding bodies is leveraged by international collaborations. Opportunities to disseminate research findings extend beyond journals of primary interest to the ophthalmic community, again providing incentives for collaborations outside the vision research community.

Limitations

As with all research, the validity of bibliometric studies varies with the completeness and accuracy of the data on which they are based. There is no perfectly complete bibliographic database of biomedical publications. *MEDLINE*®/PubMed® and *Web of Science*™ have overlapping coverage, but each provides access to a discrete set of journals; in addition, the metadata captured or added during the indexing process varies between the files and may also vary over time. The choice of *Web of Science*™ for this work was made because of the more complete author affiliation and funding information in this file, as well as point-and-click analytics for the fields of each database record and citation information and analysis. Nevertheless, the lack of subject indexing means the research must rely on either journal classification or natural language keyword searches. The former is incomplete, as this study demonstrates that approximately 40% of ophthalmic genetic articles identified with keywords were published in ophthalmology-classified journals. Natural language searching relies on divergent and creative association of important concepts in a field with terms capturing those concepts and yet involves creative discrimination to eliminate terms that are associated with a different domain.

Metadata for fields such as author affiliations and funding agencies are taken from the published articles and are subject to the vagaries of the authors. For example, funding by the US National Eye Institute is styled variously as NEI NIH HHS, National Eye Institute, NEI, National Eye Institute National Institutes of Health, NIH NEI, National Eye Institute NIH, NEI NIH, and National Eye Institute NEI; the Ministry of Education Culture Sports Science and Technology of Japan is also the Ministry of Education Culture Sports Science and Technology Japan, Japanese Ministry of Education Culture Sports Science and Technology and presumably the Ministry of Education Culture Sports Science and Technology, with no designation of country; "Ministry of Education Culture Sports Science and Technology MEXT" introduces the acronym MEXT, possibly sometimes misspelled as MEST. Thus identifying a single funder to assign credit is an uncertain task. Similarly, while the most common variants on National Eye Institute as a funding agency yielded 804 citations from recognized variants, searching for the string EY* (the truncated version of the common two-letter

identifier on NEI grant numbers) and a small set of variations on the National Eye Institute as the funding agency retrieved 1355 citations in this data set.

Conflict of Interest The author declares that she has no conflict of interest.

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3 Opportunity for Population-Based Eye Research in Asia and the Middle East: An NGO Perspective

Suzanne S. Gilbert, Thulasiraj Ravilla, and Leslie Louie

Abstract

Advances in genetics eye research are meeting and exceeding expectation. Research collaborations, rapid sharing of findings, and inclusion of emerging technologies offer tremendous opportunities. Beyond researchers per se, there is also a largely untapped opportunity for expanding the reach of population-based genetics research through engagement with the eye care NGO sector. In addition to the prospect of enabling rapid engagement with larger populations, the NGO sector can be an ally in building the capacity for research among service providers. This chapter frames this opportunity and offers recommendations to genetics researchers who seek to broaden their array of research options.

Keywords

Capacity building · Training · Population research · VISION 2020

3.1 Community Eye Health

As noted elsewhere in this volume, the past 20 years have marked a considerable increase in organized efforts to reach the unreached with comprehensive eye care. The 1999 launch of VISION 2020: The Right to Sight, the joint initiative of the World Health Organization (WHO) and the International Agency for the Prevention of Blindness [1], signaled global recognition of the scope of the unmet needs. This global initiative prioritized strategies required to address the needs and an advocacy platform to align ministries of health, nongovernmental organizations, and professional societies in the vision field.

WHO's Universal Eye Health: A Global Action Plan 2014–2019 [2] prioritizes three objectives for member state action: (1) recognize the need for generating evidence on the magnitude and causes of visual impairment and eye care services and using these data for advocacy; (2) encourage the development and implementation of integrated national eye health policies, plans, and programs to enhance universal eye health in line with WHO's framework for strengthening health systems to improve health outcomes; and (3) address multi-sectoral engagement and effective partnerships to strengthen eye health. Three key indicators include (1) the prevalence and causes of visual impairment, (2) the number of eye care personnel, and (3) cataract surgery volume.

S. S. Gilbert (✉) · L. Louie
Seva Foundation, Innovation & Sight Program, Berkeley, CA, USA
e-mail: sgilbert@seva.org

T. Ravilla
Aravind Eye Care System, Madurai, India

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These priorities plus findings regarding causes of vision impairment [3] and projections through 2050 [4] prompt a call to action and priority to improve program effectiveness. A comprehensive presentation of data and strategies is found in the IAPB Vision Atlas [5].

Through international efforts to conduct community-based prevalence studies including the initiation of the Rapid Assessment of Avoidable Blindness (RAAB) strategy [6, 7], the prevalence of conditions causing visual impairment and systematic strategies to address them has become better known. The global RAAB Repository [8] now cross-references findings from more than 320 studies meeting the stringent RAAB criteria. Population-based studies and disaggregation of service data have produced breakthroughs in recognition of service inequities and need for interventions for marginalized groups. A case in point is the finding that vision impairment occurs disproportionately in females [9] which has promoted efforts to refine strategies to reach women and girls [10, 11].

3.2 Capacity Building to Scale Service Delivery

Given the growth in visual impairment projected due to global demographic trends and insufficient service levels causing a threefold increase by 2050 in the number of people who are blind and visually impaired, [4] planners recognize the requirement to develop programs that are built to last. A systems development approach is increasingly being applied to build the platform for robust service delivery. The scope of the "ophthalmologist gap" due to lack of number of ophthalmologists and their uneven distribution around the world further exacerbates the need [12]. A growing number of service programs are working to increase services within a sustainable framework. This requires having the means to develop and manage highly skilled staff, to design and implement community-engaged interventions, and to provide high-quality surgical and clinical care that is sought by patients. As programs have become more robust, the push is for organizational sustainability of the service delivery programs including, where feasible, financial sustainability.

Several service institutions and international nongovernmental organizations that had achieved program stability experimented with reaching out to help underperforming eye hospitals to improve [13]. Capacity building has become more systematic and has grown into a leading strategy implemented by a growing number of organizations. One such organized approach is the Global Sight Initiative which today involves 100 eye hospitals in 16 countries [14].

Classically, capacity building involves developing a relationship between the more established institution (often referred to as a "mentor" and the institution seeking to improve the "mentee"). The process begins with an exploratory phase of needs assessment, followed by engagement together to determine a strategic plan and an action plan. Ongoing consultation to enact the plan focuses on improving administrative systems, training key clinical and nonclinical staff, strengthening community outreach, and advising on facilities improvements and equipment that will enhance service quality and volume. Throughout, the groundwork is laid for monitoring of key performance indicators through data collection, reporting, and feedback. Initial results in scaling cataract surgery productivity reported by the Global Sight Initiative are encouraging [15].

A critical aspect of capacity building is to ensure increase in a geographic region's Cataract Surgical Rate (CSR, number of cataract surgeries performed per one million population in a given year). Though this metric focuses on cataract surgeries, the strategies to achieve this require reaching out to larger number of patients. This, combined with the thrust for comprehensive eye care, ensures enhanced delivery of eye care. This achievement requires intelligence regarding community eye health seeking behavior or lack thereof. A landmark study conducted with Aravind Eye Care System reported that even with extensive community outreach to find people in need of eye care services, only 7% of people in need of eye care came for the local, free screening camp [16]. This finding plus observations by LV

Prasad Eye Institute and other providers lead to the development of community-based vision centers which provide primary prevention services and early referral to the corresponding eye hospital as needed [17].

3.3 Capacity Building Case: Sadguru Netra Chikitsalaya in Rural Central India

2001: Sadguru Netra Chikitsalaya (SNC) was a 350 bed eye hospital producing 20,000 cataract surgeries per year. SNC had one full-time ophthalmologist and a large number of volunteer surgeons during 3 months of the year when 85% of annual surgeries were conducted. Surgeries were provided free of charge with just 16% of the cataract surgeries being done with an intraocular lens. It was difficult to hold professional staff given local hardships such as lack of electricity and schools for children.

A series of systematic capacity building inputs were made with SNC by Aravind Eye Care System, Seva Foundation, and Orbis over a multiple year period to attract and retain staff, train managers, and install management and information systems. The immediate focus was to convert from intracapsular to extracapsular cataract surgery with IOL implants and soon to manual small incision and phaco techniques.

In 2016, to promote year-round utilization of services, SNC was coached in building a robust community eye screening program which has grown to include 43 vision centers, increasing proportion of cataract patients who are female to 50% and establishing strong financial analytics and management practices [18]. SNC's growth is not limited to cataract surgery which in 2015–2016 rose to 117,661. Between the 2 years, 2014/2015 and 2015/2016 alone, SNC's specialty surgeries in glaucoma, vitreoretina, cornea, pediatric surgeries, and orbit and oculoplasty increased 10% from 23,613 to 26,063. In 2015/2016, SNC was able to serve 40% of its patients free of charge based on cross-subsidies from paying patients. From not being able to retain any ophthalmologists, the hospital has 117 specialist doctors on staff [19].

Over time, SNC's capacity building extended into establishing postgraduate ophthalmology training programs, optometry training, and training for ancillary personnel. Coaching and assistance was provided by Seva, Aravind, and LV Prasad Eye Institute to initiate a public health library to support all of these training activities. During 2010, SNC started mentoring other eye hospitals to improve their systems and results through the Global Sight Initiative.

(End of Case)

Establishing in-house training programs is a vital component of capacity building, particularly for training Allied Ophthalmic Personnel (ophthalmic assistants, midlevel ophthalmic personnel, refractionists, surgical assistants, patient counselors, ward staff, and other members of the ophthalmologist-led eye care team). The International Council of Ophthalmology, Aravind Eye Care System, and Seva Foundation established an immersion course to enable eye hospitals to establish or expand their in-house training programs [20]. This "Eyexcel" workshop has prepared 121 eye hospital teams from 24 countries to improve their training practices.

However, capacity building does not stop at service delivery and training. There also are efforts underway by NGOs to strengthen research practice at the population and clinical level.

3.4 Research Skill Building and Partnership Development

The NGO sector has been engaged in population-based studies of the prevalence and causes of visual impairment as a tool for service program planning, development, and impact assessment. There also is a tradition of operations research to improve the use of scarce resources for service delivery such as staff, facilities, equipment, and supplies. This institutional research promotes inquisitiveness among staff who seek to reach more patients more efficiently [21, 22]. Research into the outcomes of care are conducted to

determine how service outcomes as experienced by patients can be improved [23–25]. In this era of impact evaluation and determination of return on investment, it is incumbent upon programs to master these analytical skills.

3.5 Implications for Population-Based Genetics Eye Research

As programs manage to tackle the more prevalent eye conditions and grow to provide comprehensive and specialty services, ultimate prevention through genetics research takes on increasing interest.

Developing this robust network of eye hospitals adopting best practices in the delivery of care combined with good documentation offers a fertile ground for all disciplines of research from genetics to operations research. For instance, Netra Niramay Niketan in West Bengal, India, recognized an unusually high number of albino children coming from a particular village, and this led to a genetic exploration in collaboration with Aravind Medical Research Foundation [26, 27] and others [28]. There has been another instance of a rural hospital in Bihar recognizing that they were seeing an unusually high number of infants with anophthalmia [29] and microphthalmia, a rare condition, coming from a specific region, again prompting genetic and environmental explorations.

Cross-fertilization of service delivery program findings with interests of genetics research can prove to be mutually beneficial.

Conflict of Interest All of the three authors declare they have no conflict of interest. The three authors are Suzanne S. Gilbert, Thulasiraj Ravilla, and Leslie Louie.

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4 eyeGENE®: A Model for Advancing Research of Rare, Inherited Eye Conditions Through Biobanking and Data Sharing

R. S. Parrish, K. E. Goetz, and S. J. Tumminia

Abstract

eyeGENE® is a genomic medicine initiative created to facilitate research into the causes and mechanisms of inherited eye diseases and accelerate pathways to treatments. Through collaborations with clinics across the United States and Canada, eyeGENE® recruited approximately 6400 participants. To date, eyeGENE® has returned more than 5800 clinical genetic test results to participants and has granted 22 research studies controlled access to clinical and genetic data, DNA samples, and/or individuals consented to participate in research and clinical trials. These studies have identified new genes, improved our understanding of inherited eye disease, and validated new molecular diagnostic technology. eyeGENE® can serve as a model for creating a research resource to advance the study of rare, inherited diseases.

Keywords

Data sharing · Biobank · Biospecimens · Genetics · Clinical phenotype · Common data elements · Clinical data collection

R. S. Parrish · K. E. Goetz (✉)
Ophthalmic Genetics Visual Function Branch, National Eye Institute (NEI), National Institutes of Health (NIH), Bethesda, MD, USA
e-mail: goetzke@nei.nih.gov

S. J. Tumminia
Office of the Director, NEI, NIH
Bethesda, MD, USA

4.1 Introduction

The National Ophthalmic Disease Genotyping and Phenotyping Network or eyeGENE® is a genomic medicine initiative created by the National Eye Institute (NEI) at the National Institutes of Health (NIH), a part of the United States (US) Department of Health and Human Services. The primary goal of eyeGENE® is to facilitate research into the causes and mechanisms of inherited eye diseases. To accomplish this, eyeGENE® established a patient registry, a genotype/phenotype data repository, and a DNA biobank. eyeGENE® enables access to genetic diagnostic testing for affected individuals and their families. Additionally, eyeGENE® provides research access to clinical and genetic information including images, DNA samples, and the opportunity to recruit individuals to participate in additional research studies and clinical trials.

eyeGENE® arose from vision community needs and opportunities. Thirty years ago, clinicians could do little more than identify an inherited eye disease through standard direct ophthalmoscopy, but scientific and technological advances have brought potential treatments and hope to individuals impacted by these conditions. To date, scientists have identified over 400 genes related to eye disorders that are associated with both common complex diseases as well as rare diseases [1]. This profusion of genetic information emphasizes the significant strides made in

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understanding the molecular basis of human eye diseases. These advances are paving the way for the development of gene-based therapies to treat ophthalmic genetic diseases that were once considered untreatable.

eyeGENE® has accrued over 6400 individuals and to date has returned clinical test results to roughly 75% of participants through their referring clinician. Even though the program offers this knowledge to the participant as an indirect benefit, the main goal of eyeGENE® is to facilitate research. Toward achieving this goal, researchers have used eyeGENE® to identify new eye disease-causing genes, better understand various characteristics of inherited eye disease, and validate new molecular diagnostic technology [2–7]. Through experience, the eyeGENE® Operations Working Group has learned valuable lessons about building a network to advance research of rare genetic eye diseases. Here we share many key points in patient accrual, biobanking, clinical data collection, and research analysis.

4.2 eyeGENE®: Initial Concept and Design

In the early 2000s, diagnostic DNA testing was not widely accessible to the public, and there were no concerted efforts to identify individuals who might benefit from potential gene-based treatments. The NEI addressed these needs by convening meetings with domestic and international experts in ophthalmic genetics, bioethics, genetic counseling, and regulatory requirements. These experts advocated for the establishment of a vision community resource to bring together individual genetic testing laboratories for the common goals of patient care, diagnosis, research, treatment, and education. In February 2004, a concept for this resource was approved by the National Advisory Eye Council, an external group authorized under 42 U.S.C. 284a, section 406 of the US Public Health Service Act to advise, assist, consult with, and make recommendations to the Secretary of Health and Human Services and the NEI Director on matters related to NEI activities and policies.

Over the next 2 years, the NEI funded a set of extramural vision community research grant supplements to build the national capacity for CLIA[1]-approved molecular genetic testing for ocular diseases. Concurrently, the NEI developed a web-based application to recruit participants from clinics across the United States and Canada. The application was used to collect eye examination information, images, demographic information, and molecular test results. Also, an internal Operations Working Group was created to manage the day-to-day activities and an external Steering Committee was formed to help oversee progress [8]. In 2006, eyeGENE® was officially launched. Through the next decade, eyeGENE® became well-known as an international multicenter clinical genetics research initiative and a model of partnership between the US federal government, eye healthcare providers, CLIA-approved molecular diagnostic laboratories, private industry, and extramural scientists who support a broad research constituency. As a vision community resource, eyeGENE® provides patients with greater access to diagnostic gene testing and genetic information. Clinicians and researchers also have access to diagnostic genetic testing, centralized specimen collection, and a shared database of de-identified genotype/phenotype information. Overall, this public-private partnership benefits eye healthcare providers, patients, and researchers [9].

eyeGENE® was designed as a two-stage initiative governed by human clinical protocols approved by an NIH Institutional Review Board (IRB). Stage 1 of the eyeGENE® initiative (Clinical Trials.gov Identifier NCT00378742), *National Ophthalmic Genotyping and Phenotyping Network*, *Creation of DNA Repository for Inherited Ophthalmic Diseases*, accrued samples from individuals with inherited

[1]Clinical Laboratory Improvement Amendments (CLIA) – The Centers for Medicare & Medicaid Services (CMS) regulates all laboratory testing (except research) performed on humans in the U.S. through the Clinical Laboratory Improvement Amendments (CLIA). The objective of the CLIA program is to ensure quality laboratory testing (https://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/index.html?redirect=/clia)

eye diseases, while Stage 2, *Distribution of Data and Biomaterials from the NEI*, allows the allocation of samples, data, and the contact of participants recruited through Stage 1.

When the program began, eyeGENE® screened for 20 known disease-causing genes in 9 disease categories. Currently, several hundred genes are tested through panels available through eyeGENE® Network Laboratories. As molecular testing technology evolves, so does the screening capabilities of the program. The eyeGENE® website lists summary data and shows the number of participants in each disease category and the breakdown of variants for genes tested [10]. The two largest disease categories are retinitis pigmentosa with more than 2000 participants and Stargardt disease with over 1300 participants. Nine additional disease categories have more than 100 participants, which is noteworthy given the rarity of these conditions in the North American population.

4.3 Building a Network and Participant Recruitment

One of the first strategies of eyeGENE® was to build a network of research and clinical investigators interested in diagnosing genetic eye disease through molecular testing. To achieve this, the NEI issued a Notice of NEI Administrative Supplements to Enhance Diagnostic Genotyping for Ophthalmic Diseases (https://grants.nih.gov/grants/guide/notice-files/NOT-EY-05-002.html). The Notice announced the availability of supplemental funding to ongoing NEI-funded research projects to expand and enhance molecular diagnostic testing for ophthalmic diseases. The purpose of these administrative supplements was to build infrastructure for genotype/phenotype resources that would be publicly available by assisting researchers who had an ongoing NEI-supported project related to genetic screening of inherited eye conditions to build or expand their laboratories' capacity for clinical testing. CLIA-approved laboratories that received this funding from the NEI were the first molecular diagnostic testing facilities collaborating in the eyeGENE® Network. As technology evolves, the molecular testing partners have changed and so has the funding mechanism; however, the molecular diagnostic testing services are primarily contracted outside of the NEI with commercial and academic CLIA-approved laboratories.

eyeGENE® accrued participants by allowing healthcare professionals (ophthalmologists, optometrists, neurologists, genetic counselors, etc.) with knowledge and access to the clinical details of an affected individual's inherited eye condition to enroll the patient. Because of the challenges of sample collection, the constraints on international blood shipments, and financial considerations, enrollment was open to healthcare professionals in the United States or Canada. More than 220 clinical organizations registered with eyeGENE® and enrolled one or more participants. The first enrollments were patients referred from the ophthalmic genetics branch of the NEI Clinic but quickly grew to include patients from large research hospitals and academic institutions and private practice.

In large part, clinical organization registrations were fueled by patient interest. A strong driving force for some patients to participate in the program was that they would receive "free" diagnostic testing. That is, the onus to pay for diagnostic testing, often multiple testing, was on the NEI, and not on the patient. In return, the patient agreed to participate in research with the option to withdraw at any time. The patient was responsible for the cost of their clinical eye exam and blood draw.

From 2006 to the end of 2015, eyeGENE® enrolled more than 6400 participants. Collaborating with community clinicians and large academic institutions allowed eyeGENE® to cast a wide net in finding rare eye disease patient populations as shown in Fig. 4.1. eyeGENE® participant contact information was used to map participants by enrolling clinical organization. The NEI Clinic enrolled more than 1000 (16%) participants. While the NEI Clinic had the greatest reach of any organization, most participants live relatively close to the clinic. Thirteen other clinical organizations enrolled more than 100 participants each and close to 3500 partici-

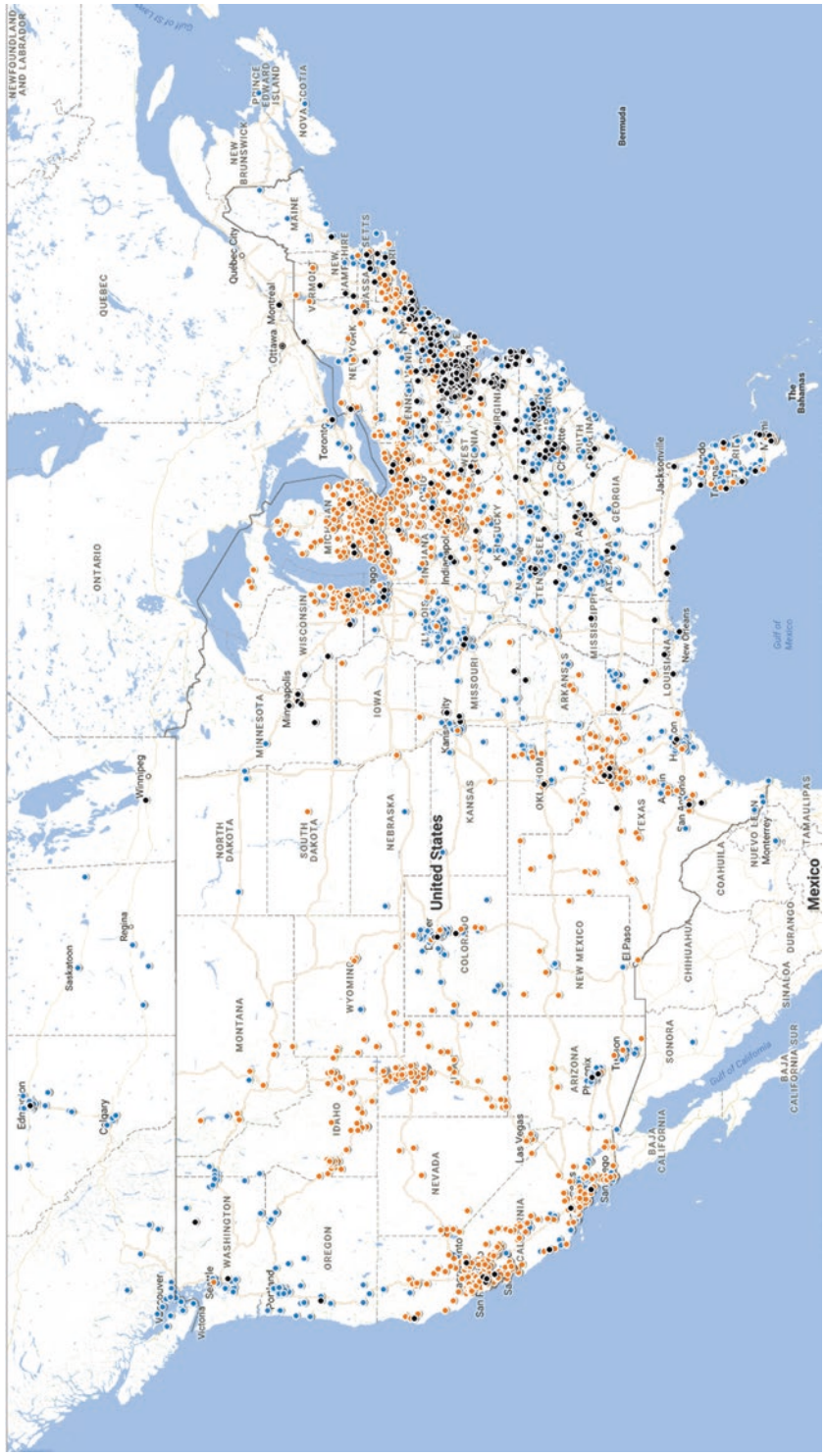

Fig. 4.1 Location of eyeGENE® participants in the continental United States and parts of Canada and Mexico, based on contact information provided during enrollment. The black points are participants enrolled by the NEI Clinic. The orange points are participants enrolled by the 13 high-enrolling clinics (each enrolled 100 or more participants). The blue points are participants enrolled by clinics that enrolled less than 100 participants

pants (54%) collectively. The remaining 30% of participants were registered by clinics that enrolled less than 100 participants, and 85 of these only enrolled one participant. Building a large, diverse network of clinicians allowed patients in every part of the United States, including rural and remote areas, to participate in eyeGENE®. One downside to this type of network is that the quality of clinical data is highly variable due to differing levels of experience with rare diseases and access to diagnostic equipment among clinicians.

4.4 Informed Consent

All eyeGENE® participants were consented in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the World Medical Association's (WMA) Declaration of Helsinki, as revised in 2008. When planning a biobank, national and institutional regulations regarding informed consent of human subjects should be consulted. Specific ethical considerations regarding health databases and biobanks can be found in the WMA's Declaration of Taipei [11].

Two routes of consenting were proposed and accepted by the NIH Combined Neuroscience IRB. The consenting method was dependent on the anticipated patient enrollment volume from an approved registered clinical organization as well as the internal regulatory policies of the enrolling institution. Clinical organizations planning to enroll large numbers of participants in eyeGENE® were likely to be engaged in additional research on those populations. Therefore, to enroll more than ten participants per year, a clinical organization was required to submit the eyeGENE® protocol for approval to their respective institution's IRB or a commercial IRB. More than 40 clinical organizations sought and received independent IRB approval of the eyeGENE® protocol. A second infrastructure was implemented for those enrolling less than ten patients per year as it was recognized that many private practices or small institutions might only see one or a small number of eligible patients due to the prevalence of these diseases. These organizations were approved to use the NIH-issued consent forms and were not expected to undergo the often lengthy and expensive IRB protocol submission process.

While the flexibility of this consenting strategy expanded eyeGENE®'s reach, it also created some difficulties. Verbally confirming the NIH consents obtained by collaborating organizations was time-consuming for NEI staff. The independent IRB-approved protocols and consents expired at different times throughout the year and ensuring that the protocols were being maintained and that valid consents were being used required vigilance from both the eyeGENE® Coordinating Center and the Laboratory. Incomplete and expired consents were received regularly, requiring follow-up, which delayed the ability of NEI staff to complete patient enrollment and provide genetic test results.

During the consenting process, eyeGENE® participants were given several options. In addition to consenting to allow their de-identified samples and data to be used for vision research, they could choose whether they wanted to receive their genetic test results or just participate in research. Most participants opted to receive their genetic results. Participants were also given the option to be recontacted in the future.

One constant in research is change, and as technology and the knowledge of human genetics expanded, so did the potential for research overlap outside of strictly vision-related elements within the original purview of the eyeGENE® protocol. For instance, some genes associated with a diagnosis covered by eyeGENE® may also be involved in pathways related to other, non-eye related conditions for which some researcher may be interested. Also, whole-genome/whole-exome or other next-generation sequencing techniques may identify genetic causes of eye disease in a participant that may not be strictly related to their clinically determined eye condition, especially since there is a high level of clinical and genetic heterogeneity in the retinal eye disease population. For these reasons, eyeGENE® is implementing a patient portal to keep partici-

pants engaged in the research endeavor. The portal includes an electronic consent to provide existing participants an opportunity to determine whether they wish to participate in a broader definition of vision research. A specific advantage of an electronic consent is that it can involve multimedia information and be interactive, which is important for providing anonymity for participants with vision impairments who may need to use assistive technology for reading. Additional benefits are reduced staff time, cost-benefit efficiencies, tools for checking participant comprehension, and electronic records management. The portal is also a useful tool for administering surveys to expand the resource. eyeGENE® participants have complained about the need for more communication, and this measure is one step to serve their desire to play a continued role in the research enterprise.

4.5 Building a Biobank

When eyeGENE® was launched, biobanking was beginning to be recognized for its value to scientific research. Prior to this time, many considered a biobank to be a freezer full of patient-derived samples often for personal research projects. Biobanking has blossomed to a scientific field of its own with organizations like the International Society for Biological and Environmental Repositories (ISBER) and the National Cancer Institute, also part of the NIH, providing science-based evidence to this expanding landscape. Extensive planning is required during the creation of a biobank, and best practices are available [12, 13]. During the planning phase, it is important to consider the purpose of the biobank, type of specimen(s) to be collected, specimen processing and storage conditions, and type(s) of research utilization among other characteristics.

eyeGENE® created a DNA biobank for rare inherited ophthalmic diseases to provide DNA coupled to de-identified phenotypic information to researchers. To accomplish this goal, eyeGENE® collected large quantities of high molecular weight DNA that could be used immediately for diagnostic testing purposes and be stored for future research needs. Instead of collecting DNA that had been extracted by an outside source, eyeGENE® chose to collect blood and process it to DNA centrally using standardized procedures in the eyeGENE® CLIA-certified laboratory. This ensured that the quality and quantity of DNA banked would be comparable and adequate for current and future needs. While saliva was considered, blood was chosen because of the higher DNA yield and it is relatively easy to access and transport.

To enroll in eyeGENE®, participants had to be willing and able to provide a blood sample of acceptable quality and volume to yield more than 50 μg of DNA. To meet this requirement, eyeGENE® requested blood (24–30 mL from adults and 7–15 mL from children) collected in K_2EDTA tubes, which are commonly used when collecting specimens to be used for PCR-based genetic testing. To control for quality, receipt and initial processing of the blood had to be completed by the eyeGENE® Laboratory within 72 h from the time of collection as it was assumed that the longer the sample was in variable transport conditions, the more degraded the DNA would become. Samples received after 72 h from the blood draw were subject to rejection. Whenever possible, a participant's blood sample was aliquoted into two or more tubes for the initial processing of sample to cell lysate. Then the tubes were extracted to DNA in separate batches and the resulting DNA stored in separate freezers. One DNA aliquot is designated as the active aliquot for distribution and stored at −20 °C. Any other DNA aliquots are designated as long-term storage and stored at −80 °C. Processing a participant's sample in multiple batches and storing the resulting DNA in separate locations greatly reduce the risk of losing an entire sample to processing errors or equipment failure.

During initial processing, 0.5–1 mL of blood was stored in one of the original blood tubes at 4 °C. These original blood samples are used during Sample Confirmation Testing (SCT), an internal QA/QC procedure that compares STR markers in the blood to those in the corresponding DNA [14]. This process is a vital step to ensure no significant laboratory errors, including misla-

beling, pipetting, or other human errors, occurred. As policy, only DNA aliquots that have been verified to match the original blood sample through SCT may be shipped out of the biobank to a Network CLIA-certified molecular diagnostic laboratory and/or to an approved researcher.

Standard operating procedures (SOPs) were created and revised for each process involved in specimen receipt, handling, extraction, and storage. The eyeGENE® Working Group shares these SOPs with anyone who has an interest in building their own capacity and standardized processes.

4.6 eyeGENE® Laboratory Sample Tracking System

Developing and maintaining records management and computer-based sample tracking systems are best practices for all biorepositories. Over time, the eyeGENE® system for records management and sample tracking evolved from a simple paper logbook and a Microsoft Access database to a complex custom-built system, hereafter referred to as the eyeGENE® Legacy system, that was informally developed in-house.

The lack of formal workflow analysis led to a system that had to be supplemented by several Microsoft Excel logbooks and paper procedure worksheets. The logbooks served to batch and track samples through processing as well as assign sample storage locations, while the procedure worksheets documented the technician, reagent information, and samples in the batch. Free-text notes were manually entered in a text box comment field for each sample listed in the Legacy system as a basic way to track chain of custody, storage conditions, and freeze-thaw cycles. This process was time-consuming, prone to human error, and not suitable to query. In addition, sample storage locations needed to be manually entered into the database, which had no data validation mechanism. This deficiency allowed entry of nonstandard formats, erroneous and duplicate locations for multiple samples.

To improve laboratory workflow, records management, and sample tracking, eyeGENE® contracted Sampleminded (now part of Exact Sciences Corporation) to customize and implement their web-based commercial laboratory information management system (LIMS). The two teams analyzed the eyeGENE® Biorepository's existing processes and systems and identified problems and workflow bottlenecks. This analysis led to the development of detailed LIMS requirements and an overall project development plan. Using eyeGENE® SOPs as a guide, existing Sampleminded LIMS tools were configured and new tools were developed as necessary. Iterations of releases were tested by the eyeGENE® team, and corrections and improvements were made before launching the production system.

Nearly all laboratory procedures are now successfully being managed by the customized Sampleminded LIMS, which has markedly increased the efficiency of the eyeGENE® Laboratory workflow. In addition to guiding users through each procedure, the LIMS has eliminated the need for paper procedure worksheets because it includes a detailed audit trail which automatically records the user, reagent information (lot number and expiration date), and batch. Using a robust barcode scanning system, the LIMS has automated chain of custody and improved sample tracking by annotating data from each workflow event and recording the location for every sample in the biorepository. The scanning system also helps prevent errors in tube transfer and storage. A reduction in manual data entry combined with the LIMS double-entry requirements and data validity checks has improved data quality (e.g., the LIMS will not allow two tubes to be assigned the same location).

A LIMS is an effective tool for automating and improving workflow, accurately recording process and sample-related data, and tracking samples. Both in-house developed and customized commercial LIMS require significant time and resources, and it is imperative that requirements are carefully generated before building or selecting and customizing a LIMS. Despite these challenges, those seeking to develop a biobank should consider incorporating a LIMS to enhance efficiency and, more importantly, to reduce

errors. Finally, it is best to consider a LIMS during the initial planning phase due to the added resources that are needed to extract data from one system and import that data into another system.

4.7 Clinical Data Collection

Often biobanks contain clinical data that correspond to samples stored in their freezers. Sometimes biobanks are created specifically to support a hospital system with a vast collection of longitudinal clinical records. For eyeGENE®, the Legacy system was developed to allow referring healthcare providers to enter clinical data about the eye health of a patient at the time of enrollment. The system was designed to support a laboratory workflow that prohibited genetic diagnostic testing of the patient's sample until the required paperwork and a minimal amount of defined clinical data was received.

Over time, the eyeGENE® Legacy system began to require integration of the data corresponding to patient samples as described in the prior section. This need for operational efficiency eventually led to all the sample-related data becoming part of the Legacy system. To keep an acceptable turnaround time from enrollment to genetic testing to the return of genetic results, most of the system development resources were diverted to enhancing features of the laboratory aspects of the Legacy system. Additionally, in 2010 with the research goal of eyeGENE® in mind, the requirements of the Legacy system mandated one more crucial feature – researcher access to de-identified clinical and genetic data. The combination of three major core functions, i.e., clinical data capture, laboratory management (LIMS features), and research access, coupled with advances in technology and other system constraints, eventually led to the retirement of the Legacy system as it could no longer keep pace with other, cheaper products available "out of the box" or with some customization.

One fundamental decision in the development of the Legacy system was the decision that data collection would be organized by disease. This would end up being a poor design choice even though the issues could not have been anticipated at the onset of development planning. Upon enrolling a patient, the referring healthcare provider would select the patient's clinical diagnosis. A clinical capture report form would then be presented to complete enrollment. Each form differed based on the diagnosis and comprised roughly 10 to 15 questions that experts, solicited by the eyeGENE® Steering Committee, decided represented the core clinical symptoms of that disease presentation. These questions were dubbed "minimal clinical criteria" and were used to validate the patient's diagnosis and the requested genetic test(s). Meeting these criteria also served to justify the federal support for the clinical genetic testing costs. The program started with 9 disease categories and grew to more than 35 categories over the course of 10 years, and each time a new diagnosis was added, an expert was identified to add new minimal clinical criteria questions to the system.

Two critical hurdles arose from using this strategy. First, through the years over 300 questions were created to describe the phenotypes of eyeGENE® participants. With only 35 disease categories, most retinal diseases, there was a great deal of overlap of descriptive terms. For example, there were at least six distinct questions that asked the referring clinic to describe fundus appearance. Each question contained a different set of answer selections. The second problem was that the diseases included in eyeGENE® were clinically heterogeneous, meaning that there was clinical overlap among conditions. It is sometimes difficult to distinguish one diagnosis from another based solely on clinical symptoms. Through genetic testing, eyeGENE® has found that the genes associated with a diagnosis also presented with a significant amount of heterogeneity. For instance, the *RDS* gene can be associated with cone-rod dystrophy, pattern dystrophy, Stargardt disease, or retinitis pigmentosa. These issues are critical to note for anyone wishing to collect this type of data as it directly impacts how or if data sets can be combined later.

Several institutions are currently establishing similar systems using a disease category approach similar or identical to that originally used by eyeGENE®. While it is gratifying and validating to know that the data collection by disease approach used in eyeGENE® in 2006 was logical and practical as others are now implementing similar methods, the issues and challenges described above should give pause to the vulnerabilities of this approach. Creating a clinical (phenotype) capture tool that siloed data on questions related solely to diseases and clinical diagnoses with profound phenotypic and genotypic heterogeneity made research query, filtering, and analysis cumbersome and prone to error. Once the focus of eyeGENE® moved from data collection to data sharing, the existing Legacy data infrastructure was too embedded to be changed. Instead, a new approach was necessary.

In 2015, as part of a larger NIH effort to standardize disparate data, eyeGENE® began working with the Lister Hill Center at the NIH National Library of Medicine (NLM) to distill the existing eyeGENE® 300 clinical questions into a more manageable set of questions. The goal was to create a resource for clinical data collection to describe the phenotype of patients with inherited eye conditions to be used throughout the vision community, not simply eyeGENE®. This could allow for broad data sharing on a global scale. The eyeGENE®-NLM Working Group began by combining like terms, expanding lists of potential permissible values, and aligning overlapping or similar terms. Then each unique item was transformed into a structured LOINC® term, and the appropriate data type and parameters were described. LOINC® is an international coding system developed in 1994 by the Regenstrief Institute to meet the growing need for common laboratory and clinical terminology. It sets universal standards for classifying and identifying clinical and laboratory observations and measurements related to electronic health records. LOINC® enables the exchange and aggregation of clinical results for care delivery, outcomes management, and research by providing a set of universal codes and structured names to unambiguously identify measurements and observations enabling interoperable data exchange. For each concept, LOINC® contains other details, such as synonyms, units of measure, and carefully crafted descriptions. LOINC® is freely available as well as widely accepted and utilized. It is also translated internationally. The eyeGENE®-NLM Working Group also created panels of similar data elements in LOINC®. For instance, a panel was created for tonometry groups, including the type of tonometer used for the exam along with elements to capture the intraocular pressure in each eye. These terms can be found at the NIH CDE Repository under the NEI heading [15].

Common data elements (CDEs) and ontologies should be carefully selected when embarking on a clinical data capture project to ensure reproducibility and scientific rigor. It is important to take time to carefully consider the level of detail that a project requires [16]. For example, in some cases, research users may only need to know the presumed clinical diagnosis, while in other cases, the specific details of how that diagnosis was derived are needed. In some studies, it may be enough to know whether a patient had an abnormal electroretinogram (ERG), whereas in other studies, it will be important to know the specific ERG wave values and times. One final note on data elements is the concept of "flavors of null." Essentially, it is necessary to distinguish between a negative response, an unknown response, and a blank field. For example, if the data element prompts for "gross defects of the left eye," potential answers should include none, unknown, and possibly "other" field where a user can enter text if something is drastically different from the answer choice options presented.

Use of CDEs and ontologies will allow users to capture and compare clinical research data methodically, lowering the barrier for comparative data analysis [16]. Worldwide adoption of set standards across all disease categories for recording clinical encounters will allow for better data mining and cross comparison of data sets across a variety of studies and disciplines.

4.8 Community Access to eyeGENE® Data and/or Biospecimens

In 2010, eyeGENE® reached the milestone of 1000 participants. This number was deemed sufficient to allow researchers access to the rare disease data and sample resources. Access was designed to be as broad as possible, including international research laboratories and commercial entities. Requests can be made for a portion of DNA samples stored within the eyeGENE® Biorepository, access to de-identified genetic and clinical data, and/or to request that the eyeGENE® Coordinating Center contact the participants for enrollment into additional studies. Initial recontact with the eyeGENE® participant is made through the eyeGENE® Coordinating Center to determine interest so participants do not feel pressured into joining research studies. Interested investigators must submit an application, including a research proposal and IRB/ethics board review or exemption, to be considered for resource access. All researchers must sign a Material Transfer Agreement/Data Usage Agreement in which they agree to share all data with eyeGENE® with the intention that secondary research outcomes will eventually be made available to the public.

In late 2015, as the eyeGENE® Legacy system was retired, a new solution for vision community data access was needed. The NEI partnered with the NIH Center for Information Technology to utilize Biomedical Research Informatics Computing System (BRICS; https://brics.cit.nih.gov/), a bioinformatics platform that supports end-to-end life cycle support of research. It includes a set of six software modules that provide both web-based functionality and downloadable tools to support data definition, data contribution, and data access for continuous research use. BRICS coordinates the collection and quality assurance of data in standardized dictionary formats (CDEs) that enable query and distribution of aggregate data for acceleration of eyeGENE® research by the research community.

At the time of this writing, eyeGENE® has received 24 applications for access to the eyeGENE® Research Resource. A full list of studies as well as publications are listed on the eyeGENE® website (eyegene.nih.gov). eyeGENE® has been successful in aiding researchers to identify novel genetic causes of retinal conditions, testing sporadic cases of retinal degeneration with new technologies, genotype-phenotype correlation studies, molecular modeling of pathogenic variants, recruiting for rare variant-induced pluripotent stem cell studies leading to the development of therapies, enrollment of treatment trials, and development of machine learning technologies to diagnose retinal degenerations in rural areas, among others. The diversity of these studies demonstrates the breadth and reach of eyeGENE® as a valuable resource to the vision community and individuals with inherited eye disease.

4.9 Conclusions

eyeGENE® serves as a model for advancing research of rare, inherited eye conditions through biobanking and data sharing. It creates a continuous bench to bedside life cycle, with opportunities for partnership and collaboration among organizations, both domestic to the United States and abroad. Experience has shown the value of data standardization, not just the use of ontologies but also of CDEs. eyeGENE® readily shares protocols, policies, and procedures upon request.

Global interest in the program has led to a pilot of eyeGENE® International, an umbrella organization of international efforts using similar protocols and operating under similar standards and policies. Benefits to participating in eyeGENE® International is the ability to leverage local investment, work on problems of local concern through collaboration, have access to metadata, and increased patient participation in gene-based trials.

eyeGENE® has demonstrated a successful public-private partnership and has facilitated the evolution of ophthalmic care by integrating molecular genetics into standard of care for eye health professionals. The Network's clinical and genetics expertise, evolution of technology coupled with a DNA biorepository, de-identified

database, and individuals affected by inherited eye diseases are powerful tools for current investigations in human genetics and genomics and for the development of gene-based personalized medicine treatments. Now eyeGENE® intends to maximize this expertise by focusing its efforts on enhancing the research enterprise through data accessibility and sharing. Maximizing research efforts can be best achieved through data standardization and a willingness by the vision community to commit to data sharing. Worldwide adoption of set standards across all disease categories for recording clinical encounters will allow for data mining and cross comparison of data sets across a variety of studies and disciplines.

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All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

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5 Inherited Ocular Disease in the New Zealand Māori: Novel Genetic Mechanisms and Founder Effects

Andrea L. Vincent

Abstract

New Zealand is a small island nation at the bottom of the South Pacific, with a population of 4.7 million, and the largest Polynesian population in the world. 15% identify as Māori and 7% as Pacific peoples, and the spectrum of inherited eye disease encountered in this population varies from that seen in those identifying as NZ European. Keratoconus is more common, while primary open-angle glaucoma is rare. A number of founder pathogenic variants have been elucidated in autosomal recessive ectopia lentis, and a common *PDE6B* variant caused up to 16% of autosomal recessive inherited retinal disease in the Māori population. Although many of those with inherited retinal disease remain genetically uncharacterised, research to date shows a range of novel variants in many genes. Understanding the population-specific genetic disease spectrum and clinical phenotypes, as well as a knowledge of regional ancestry and iwi (tribe), aids in simplifying the diagnostic process.

Keywords

Māori · Pacific peoples · Genetic testing · Inherited retinal disease · *PDE6B* · *ADAMTSL4*

5.1 Introduction

New Zealand is a small island nation situated at the bottom of the South Pacific, with an estimated population of 4,793,700 (2017, Stats NZ, www.stats.govt.nz). With migration, New Zealand has an increasing ethnically diverse population, with 15% identifying as Māori, and also the largest concentration of Pacific peoples, recorded as 260,000 (7%) in the 2013 census (Stats NZ). The Māori people of New Zealand were the first inhabitants, with their arrival from Eastern Polynesia (Cook Islands, Tahiti, Hawai'i) estimated to occur around the year 1280 [1]. Based on genetic investigation, it is believed the Eastern Polynesians descended from Taiwanese Aborigines, with subsequent mixing with Melanesians and European ethnic groups [2, 3]. Of Polynesians, the Māori represent a relatively new population genetically (about 35 generations) and have the smallest signal of external relationship, consistent with extensive genetic drift [2]. It has been hypothesised that in the migration through the Pacific, the seafaring ancestors would likely have been subject to mul-

A. L. Vincent (✉)
Department of Ophthalmology, New Zealand National Eye Centre, Faculty of Medical and Health Science, University of Auckland, Auckland, New Zealand

Eye Department, Greenlane Clinical Centre, Auckland District Health Board, Auckland, New Zealand
e-mail: a.vincent@auckland.ac.nz

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tiple founder effects, and consistent with this, small numbers of haplotypes and reduced genetic diversity have been demonstrated [4].

This chapter will provide an overview of the spectrum of inherited eye diseases observed in the New Zealand population, specifically discussing the findings and genetic associations seen in the New Zealand Māori and Pacific peoples, based on study data, publications, and observations.

Identification of unique genetic variants in this specific population adds a further layer of complexity in determination of pathogenicity. This population is underrepresented in databases of human variation; therefore, the frequency of any allele of interest needs to be determined in an ethnically matched control population.

Investigation of genetic variants in these populations requires an understanding of, and sensitivity to, cultural beliefs and customs. Our studies have all been undertaken with institutional ethical approval, including review by the Auckland District Health Board Māori Research Review committee. Our research follows the principles of the Treaty of Waitangi and follows the tenets of the Declaration of Helsinki. All participants have provided informed consent.

5.1.1 Cornea

5.1.1.1 Keratoconus

Keratoconus is a progressive corneal dystrophy leading to a characteristic pattern of thinning and bulging of the cornea (ectasia), with induced irregular astigmatism. Onset is usually in the teens, and the incidence within the general population is estimated at 1 in 2000 [5]. In NZ, keratoconus is disproportionately the major indication for corneal transplantation, accounting for 41% of keratoplasties over a 10-year period and 67% of all paediatric surgeries [6]. This compares with rates of 11.4–28.8% in the USA and France [7–9]. The incidence is thought to be higher in the Māori and Pacific populations; based on corneal topographic values, suspected keratoconus in Māori/Pacific students was 26.9% compared with 12.9% in NZ European (p = 0.0014) [10] and 31% of affected Māori/Pacific peoples reporting a positive family history of keratoconus [11]. Keratoconus is a complex disorder with putative genetic and environmental contributions [12]. The visual system homeobox gene 1 (*VSX1*) is one gene implicated in this disease, but in our study, no pathogenic variants were detected in 26 Polynesian probands [13]. Another gene, *ZNF469*, is associated with the autosomal recessive disorder Brittle cornea syndrome, and in genome-wide association studies (GWAS), common variants in this gene play a role in determination of central corneal thickness and keratoconus [14]. In a NZ population, of which 49% were Polynesian, a large number of heterozygous variants in *ZNF429* were detected, including in 12 of the 21 Polynesian probands [15]. Two of the variants (p.(R2129K), p.(G3415V)) were absent or very rare in databases of human variation, and predicted to be harmful, but were present in a higher frequency in the control Polynesian population. Five probands carried two *ZNF469* variants, including three Polynesian families, but segregation was not complete in all families. In other populations, variants in this gene have not been shown to be of significance to disease pathogenesis. A larger study is required to determine if variants in this gene may explain, in part, the higher prevalence of keratoconus in this population.

5.1.2 Corneal Dystrophies

Many of the corneal dystrophies have been genetically characterised [16], with a consensus classification established, and updated, by the International Committee for Classification of Corneal Dystrophies (IC3D) [17].

In our studies probing the genetics of corneal dystrophies, the prevalence of disease is not clearly different from the general population; however our numbers are small. In individuals of Polynesian ethnicity, probing the *TGFB1*,

VSX1, and *ZEB1* genes, we have not identified the genetic cause for disease (stromal fleck $n = 1$, posterior polymorphous dystrophy $n = 3$) [13, 18–21].

5.1.3 Anterior Segment Dysgenesis

A NZ Māori woman had a historical diagnosis of aniridia, and congenital glaucoma, but on presentation had an enucleated right eye and extensive surgeries to the remaining eye, making clear phenotyping difficult; however we excluded a *PAX6* mutation. She subsequently gave birth to a son, affected with posterior embryotoxon and iris hypoplasia. A novel *PITX2* missense variant, c.344G>A; p.(Arg115His) at a highly conserved residue in the homeobox domain, was identified, which segregated with disease in the family [22]. It was predicted to be pathogenic, and not present in 100 ethnically matched alleles. This pathogenic variant segregated with disease in the family.

5.1.4 Autosomal Recessive Ectopia Lentis

Isolated, non-syndromic ectopia lentis attributable to mutations in the *ADAMTSL4* gene (AREL) has been observed in multiple populations, with a common 20 base pair deletion described in the European population, with evidence for a founder mutation over 4000 years ago [23]. In a cohort of 11 AREL patients, we identified a recurrent population-specific variant, c.2237G>A, p.(Arg746His) [24]. This variant was present in 81% (9/11) of probands with an early onset (under the age of 5 years) of AREL (Fig. 5.1). 77.8% (7/9) of these probands were Polynesian (Cook Island Māori $n = 5$, NZ Māori $n = 2$), and the remainder were NZ European. All individuals with this variant, regardless of ethnicity, shared a common haplotype, suggestive of a founder effect. This pathogenic variant had previously been reported once, in Turkey [25], and we hypothesised that it is possible this variant travelled from the Northern Hemisphere and was introduced into a small community by European sailors in the early 1800s.

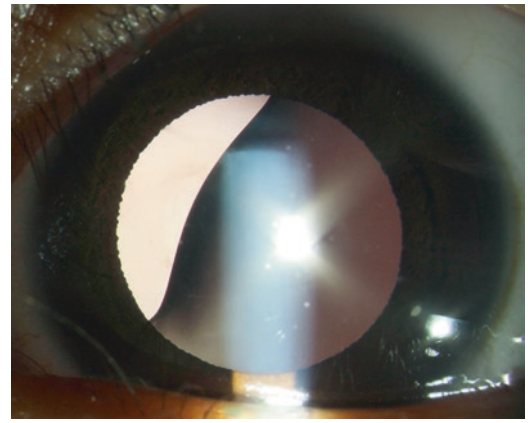

Fig. 5.1 Autosomal recessive ectopia lentis. Slit lamp photo demonstrating lens subluxation in autosomal recessive ectopia lentis in a patient homozygous for *ADAMTSL4* c.2237G>A, p.(Arg746His)

5.1.5 Glaucoma

Primary open-angle glaucoma is not common in the NZ Māori population. In an assessment of the demographics of glaucoma in 567 individuals in NZ, Māori and Pacific peoples were underrepresented for all types of glaucoma except primary angle closure [26]. In our series of 115 POAG patients in which we screened known glaucoma genes (*MYOC*, *CYP1B1*, *OPTN* and *WDR36*), none were from these ethnic groups [27]. A recent case report describes the first recorded observation of pseudoexfoliation syndrome (PXF) and glaucoma in a Māori male [28]. A previous study in Rarotonga, Cook Islands, observed PXF in 3 of 1000 subjects examined [29]. *LOXL1* variants were not studied in any of these reports.

5.1.6 Retinoblastoma

In a retrospective audit of retinoblastoma genetics seen through the Auckland Hospital Ophthalmology service, for a 5-year period from 2003 to 2008, we identified 20 patients, including

1 family, with a 100% mutation detection rate [30]. Four individuals were Māori (20%), and germ line mutations accounted for 75% of their disease, and a further 3 patients were Pacific peoples (15%). Disease in two Māori patients and one Cook Island Māori patient was attributed to a de novo germ line mutation. Although the numbers are small, and therefore not statistically significant, the higher percentage of this disease in these ethnic groups may indicate a predisposition to de novo mutations or heritable disease, as has been shown with US Latino patients with retinoblastoma, compared to non-Latinos [31].

5.1.7 Inherited Retinal Dystrophies (IRD)

In 2009, the New Zealand Registry of Inherited Retinal and Optic Nerve Disease was established, with all participating patients recruited from the Ocular Genetic Clinic, at the Eye Department, Greenlane Clinical Centre, Auckland District Health Board. This is a tertiary referral clinic, with patients from all over NZ referred for assessment. All individuals in this public hospital clinic are extensively phenotyped: history, pedigree, visual acuity, dilated slit lamp and fundal examination, OCT, fundus photography and fundus autofluorescence, and, if possible, electrophysiology. Genetic testing is initiated at the end of the consultation if appropriate, and only at this time is the invitation to participate in the IRD database given and informed consent obtained to enrol. Participation means that if no genetic diagnosis is made at commercially available testing facilities, the option to explore the underlying genetic cause for disease on a research basis is permitted. To date there are over 700 probands and family members. This is a valuable tool to determine the incidence, nature, and spectrum of different IRD phenotypes and genotypes in New Zealand.

Commercially available testing has evolved over the years; however the cost of testing is the main determinant in a publically funded eye clinic. Asper Ophthalmics in Estonia offers a microarray platform, which uses an APEX-based methodology, tested for known mutations in known genes on a "disease" microarray, e.g. autosomal recessive retinitis pigmentosa (ARRP) or autosomal dominant RP (ADRP). These traditionally have had a low yield in our population. More recently their technology has been updated to incorporate a next-generation sequencing (NGS) strategy around the regions of known mutations, but still does not sequence the gene(s) in its entirety. Specific genes have been genotyped over the years at a variety of institutions including the Carver Lab, Iowa, and the Molecular Vision Laboratory (MVL, formerly Casey Eye Institute), Portland, Oregon, as well as the Genomic Diagnostics Laboratory, Manchester Centre for Genomic Medicine, Manchester, UK, for X-linked RP genes. Newer technologies such as NGS targeted disease panels are available, with reducing prices, allowing more targeted and comprehensive screening of candidate genes, e.g. the macular panel at MVL costs $500USD and fully sequences 13 genes, including *ABCA4*, and includes the intronic regions. Retinal disease panels incorporating hundreds of genes are available but – at the time of writing – are still cost-prohibitive for our public health system.

The yield for a positive genetic diagnosis is low in the Māori and Pacific peoples' cohort using the microarray panels (discussed under individual diseases below). Using a NGS targeted retinal disease panel (105 genes, Manchester Centre for Genomic Medicine), we undertook analysis in a cohort of 28 Māori and Pacific peoples with IRD (Vincent et al., IOVS 2016:57;12 ARVO e-abstract 3157). The diagnosis in these patients was autosomal recessive rod-cone dystrophy (ARRP n = 15), dominant RP (ADRP n = 2), Leber congenital amaurosis (LCA n = 3), maculopathy (n = 4), or cone/cone-rod dystrophy (CORD n = 4). 21 unique, pathogenic variants (12 novel) were observed, allowing a definitive genetic diagnosis in 16/28 (57%) cases. Homozygosity was seen for four (*PDE6B, RD3, SPATA7, PROM1*). All LCA and ADRP cases were solved. Two ARRP cases had mutations in ADRP genes. Sixty percent of ARRP cases remain unsolved [32].

5.1.8 Leber Congenital Amaurosis/ Early-Onset Severe Retinal Dystrophy (LCA/EOSRD)

Childhood blindness due to IRD carries a significant social and economic burden and is estimated to affect 3–4 children in 10,000. LCA and EOSRD are at the severe end of this spectrum of disease, with children presenting at birth, or in the first few years of life with poor vision, roving eye movements, and non-recordable retinal electrical responses.

Of the estimated 56–140 children affected with LCA/EOSRD in NZ, we have identified 41 children from the database, for which 25 have no genetic diagnosis. The genetic cause for disease is identified in 16 individuals from 14 families, with mutations in *CRB1* ($n = 5$ probands), *RPE65* ($n = 2$), and singletons for *GUCY2D, CRX*, *LCA5*, *SPATA7*, *TULP1*, *PROM1*, and *RD3*. Four of these probands are NZ Māori/Pacific peoples, with pathogenic variants observed in *LCA5*, *RD3*, *RPE65*, and *SPATA7*, of which novel changes were identified in 75% using a NGS targeted panel (105 retinal genes) in 2013 (Vincent AL et al., IOVS 2016.57:12 ARVO E-Abstract 3157) (Table 5.1 and Fig. 5.2).

5.2 Rod-Cone Dystrophies

5.2.1 Autosomal Dominant Rod-Cone Dystrophy (ADRP)

The NZ IRD database includes 48 families with presumed ADRP, including 4 Māori, 1 Tongan, 1 Niuean, and 1 Samoan. A genetic diagnosis has been made in 26 (54%) of the entire ADRP cohort. In the Māori/Pacific peoples' cohort, the genetic cause has been identified in three families, in *IMPDH1*, *PRPF31*, and *GUCA1A* (Table 5.1).

Patient #5: A 32-year-old Māori male had an onset of his symptoms at primary school and by age 27 had 6/60 vision OU (Fig. 5.3a, b). His father was affected, and a previously reported pathogenic variant in *GUCA1A* c.149C > T, p. Pro50Leu [33], was identified that segregated with disease in the family.

A 19-year-old Samoan female (*Patient #6*) described an onset of symptoms, particularly nyctalopia, from the age of 8 years. Reportedly her brother, mother, and maternal grandmother also had similar symptoms, in particular night vision difficulties. On examination her BCVA was 6/7.5 OU, with relatively featureless fundi with peripheral atrophy, significant vessel attenuation, sparse pigment and optic disc pallor, and bilateral cystic maculopathy (Fig. 5.3c–f). The presumed diagnosis was autosomal dominant (AD) rod-cone retinal dystrophy. NGS identified a rare novel missense mutation in *PRPF31*, c.682G>C, p.(Ala228Pro), and predicted to be pathogenic. A second pathogenic variation in *IMPG2* (c.331C>T, p.(Arg111*)) previously undescribed was also present. *IMPG2* is associated with both recessive and dominant vitelliform macular dystrophy (VMD) [34].

Patient #7: A 28-year-old Maori male had nyctalopia for all of his life, with subsequent restriction of visual fields and gradual deterioration of central vision in his early 20s. His mother (Ngati Putenga Hauraki) was also affected. On examination, BCVA was NPL OD from trauma, 6/21 OS, with a diagnosis of presumed AD rod-cone dystrophy (Fig. 5.3g, h). The NGS panel identified a previously identified *IMPDH1* disease-causing variant c.968A>G, p.(Lys323Arg).

5.2.2 Autosomal Recessive Rod-Cone Dystrophy (ARRP)

There are 117 probands with presumed ARRP in the NZ IRD database, which includes those with sporadic disease, no other affected family members, and no known consanguinity. The X-linked RP genes have been excluded in many of the affected sporadic males. Thirty-one of the probands (26%) are of Māori or Pacific peoples' ethnicity (Māori $n = 17$, Samoan $n = 9$, Cook Island Māori $n = 3$, Niuean $n = 1$, Tongan $n = 1$). The diagnostic yield in this cohort using the Asper

Table 5.1 Genetic variants identified in inherited retinal dystrophies in the NZ Māori and Pacific Peoples

		Result				Allele frequency			Protein Prediction			
ID	Ethnicity	Gene	cdna	Zygosity	Protein	EVS	Exac	1000G	PolyPhen-2 (score)	Mutation taster (p-value)	SIFT (score)	Previously reported
AR LCA												
1	Māori	*LCA5*	c.194delC	het		0	0	0	N/A	Disease causing (1)	N/A	No
		LCA5	c.103C>T	het	p.(Arg35*)	0	0.00002471	0	N/A	Disease causing (1)	N/A	Yes
2	Tongan	*RD3*	c.127C>T	Hom	p.(Gln43*)	0	0	0	N/A	Disease causing (1)	N/A	No
3	Samoan	*SPATA7*	c.738_739dupAA	Hom		0	0	0	Probably damaging (0.992)	Disease causing (1)	Deleterious (0.00)	No
4	Māori	*RPE65*	IVS1+5G>A	Hom	Splice	4/13002	0.00009094	0.0002	Splice			Yes
ADRP							0.001203					
5	Māori	*GUCA1A*	c.149C>T	het	p.Pro50Leu	13/12993	0.00001652	0	Benign (0.32)		Tolerated (0.1)	
6	Samoan	*PRPF31*	c.682G>C	het	p.(Ala228Pro)	0	0	0	Probably damaging (1.0)	Disease causing (0.9999)	Deleterious (0.03)	No
		IMPG2	c.331C>T	het	p.(Arg111*)	0	0.00001652	<0.01	N/A	Disease causing (1)	N/A	No
7	Māori	*IMPDH1*	c.968A>G	het	p.(Lys323Arg)	0	0	0	Benign (0.370)	Disease causing (0.999)	Deleterious (0.00)	Yes
ARRP												
8	Māori	*PDE6B*	c.2197G>C	Hom	p.(Ala733Pro)	0	0	0	Probably damaging (0.99)	Disease causing (1)	Deleterious (0.00)	No
XLRP												
9	Māori	*RP2*	c.945_946insT	Hemi	p.(Asn316*)	0	0	0	Insertion, premature termination			No
10	Samoan	*RPGR*	c.283G>A	Hemi	p.Gly95Arg	0	0	0	Probably damaging (1.0)	Disease causing (0.999)	Deleterious (0.00)	No

11	Māori	*RPGR*	c.1236_1239delAAGA	Hemi	p.(Glu414Glyfs*10)	0	0	0	Deletion premature termination			No
		C1QTNF5	c.583dupG	het	p.(Ala195Glyfs*8)	0.00%	0	0.001	Insertion, premature termination			
12	Māori	*RPGR ORF15*	c.2360delA	Hemi		0	0	0				No
Maculopathy												
13	Māori	*ABCA4*	c.5584G>T	Hom	p.(Gly1862Cys)	0	0	0	Probably damaging (1.0)			No
14	Māori	*CRX*	c.774T>G	het	p.(Tyr258*)	0	0	0	Premature termination			No
15	Māori	*RP1L1*	c.133C>T.	het	p.(Arg45Trp)	1/12603	0.00002	0.00023	Probably damaging (1.0)	Polymorphism	Deleterious (0.01)	Yes
Cone-rod												
16	Māori	*BBS9*	c.205C>A	het	p.(Leu69Ile)	0	0	0.00002	Probably damaging (0.968)	Disease causing (0.999)	Deleterious (0.00)	No
		BBS9	c.1014_1015delinsTT	het	p.(Leu338_ His339delinsPheTyr)	0	0	0	Unknown			No
17	Māori	*PROM1*	c.1354_1355insT	Hom	p.(Y452Lfs*13)	3/12253	0.00015	0.00245	Unknown	Disease causing		Yes

Hom Homozygous, *het* Heterozygous, *hemi* Hemizygous, *EVS* Exome variant server, *EXAC* Exome aggregation consortium

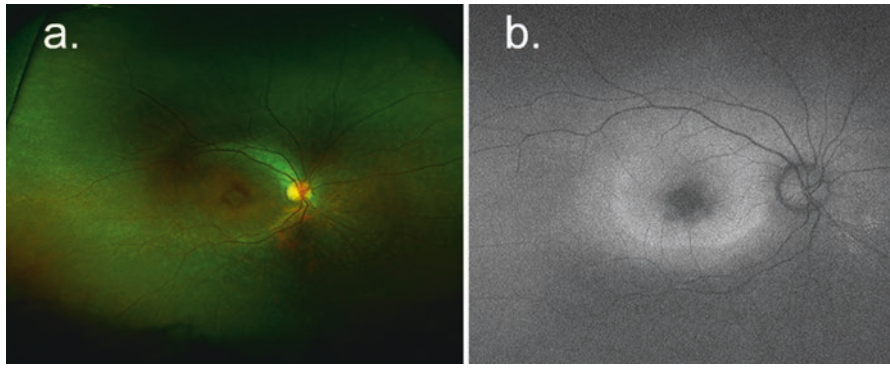

Fig. 5.2 Retinal images of an 18-year-old Māori female with Leber congenital amaurosis (Patient #4) homozygous for *RPE65* IVS1+5G>A. (**a**) Optos widefield fundus photo showing mottling of the retina with mild vessel attenuation, but no pigment, and (**b**) fundus autofluorescence showing a paramacular ring of hyperautofluorescence

ARRP microarray was zero, and a causative genetic diagnosis has only been possible using the NGS targeted disease panel as described in our recent publication [32].

A novel homozygous *PDE6B* variant, c.2197G > C; p.(Ala733Pro), was initially identified in four probands (#8) on the NGS panel and subsequently in a further six probands, based on similarity of the retinal phenotype and their iwi (tribe). Nearly all probands came from the Northern region of the North Island (Iwi Ngā Puhi) or the Gisborne, East Cape region (Iwi Ngāti Porou). Nearly 200,000 (30%) of NZ Māori identify with these two iwi. Based on our observed minor allele frequency of 0.0076, and assuming Hardy Weinberg equilibrium, we would only expect 11 affected individuals, and we have already identified 10. However, the carrier frequency is likely higher within certain iwi, and this *PDE6B* variant may account for 16% of recessive IRD in Māori.

The phenotype across all affected individuals was fairly consistent, with onset usually described around the age of 20 with nyctalopia, and the fundus appearance showing a fairly lacy pattern of mid-peripheral bone spicule pigmentation, and often a bullseye maculopathy (Fig. 5.4).

A careful history, pedigree ascertainment, and knowledge of familial iwi in a NZ Māori patient presenting with a rod-cone retinal dystrophy, and familiarity with the retinal appearance of the *PDE6B* variant, should always be considered and potentially simplify the diagnostic process, i.e. genetic testing for the single *PDE6B* variant can be initiated instead of a more expensive, multigene NGS panel or array.

PDE is a protein that has a high concentration in the peripheral membrane of retinal photoreceptors and integral to the phototransduction cycle. It consists of two catalytic subunits, PDE6A and PDE6B, and two gamma inhibitory subunits. This protein reduces the level of cyclic guanine monophosphate (cGMP) by hydrolysation, thereby resulting in channel closure in response to light [35, 36]. Mutations of the *PDE6B* gene result in accumulation of cGMP due to a dysfunctional PDE protein, leading to photoreceptor cell death [37, 38]. Although autosomal recessive rod-cone dystrophy is the most common phenotype described in association with mutations in this gene, a CSNB (congenital stationary night blindness) phenotype is also described. The majority of pathogenic variants occur within the C terminal catalytic terminal domain, including p.Ala733Pro, and lead to complete loss of enzymatic activity and subsequent accumulation of cGMP. The intracellular build-up of cGMP is known to cause photoreceptor toxicity.

By understanding the pathophysiology, it is feasible to target an aberrant process created by the pathogenic variants in the *PDE6B* gene. Using animal models, *rd1* and r*d10* mice, treated with PARP inhibitors and cGMP analogues, Sahaboglu *et al*. demonstrated a reduction in cGMP accumu-

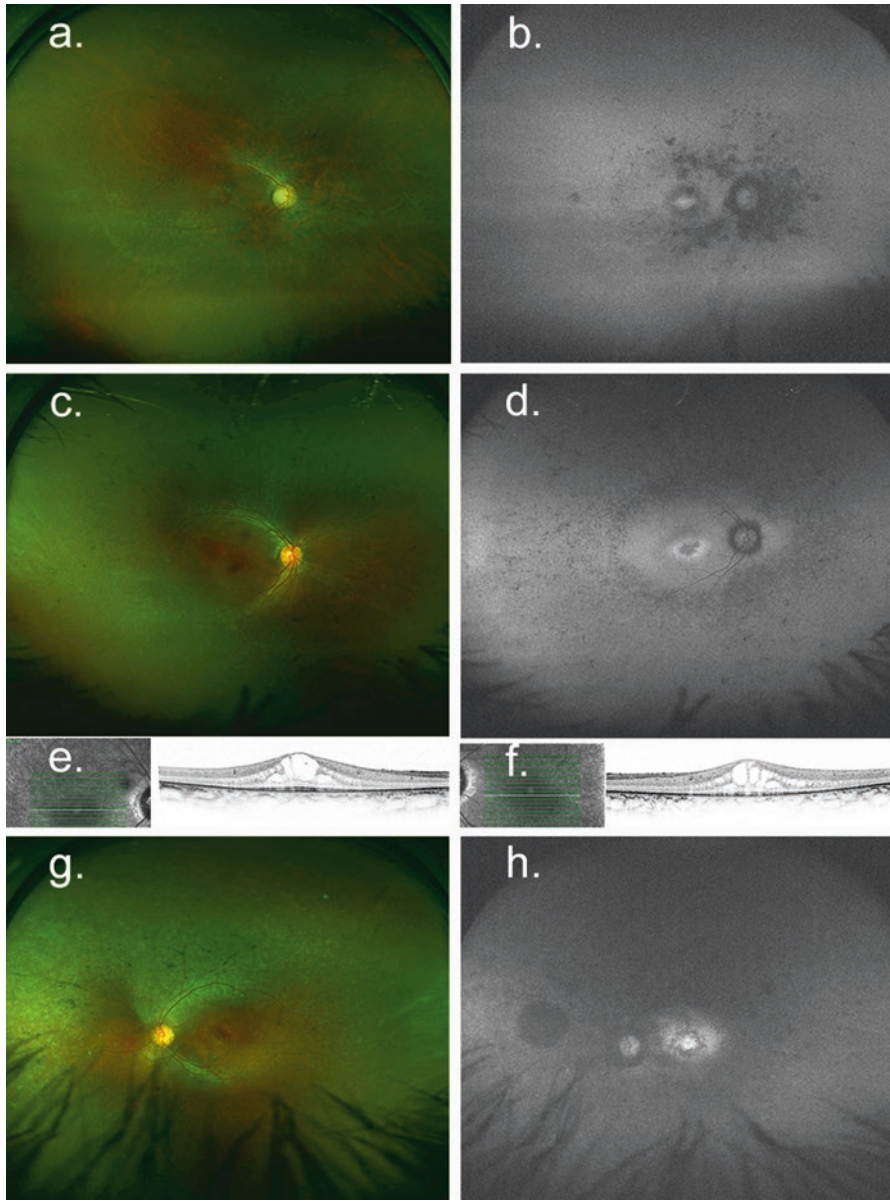

Fig. 5.3 Clinical features of autosomal dominant rod-cone dystrophies. *GUCA1A* c.149C>T, p.Pro50Leu (Patient #5), Optos widefield photo OD (**a**) and fundus autofluorescence OD (**b**). *PRPF31*, c.682G>C, p.(Ala228Pro) (Patient #6) Optos widefield photo OD (**c**) and fundus autofluorescence OD (**d**) and OCT images of coexisting cystic maculopathy (**e**) OD and (**f**) OS, with loss of photoreceptors outside the subfoveal region. *IMPDH1* c.968A>G, p.(Lys323Arg) (Patient #7) Optos widefield photo OS (**g**) and fundus autofluorescence OS (**h**)

lation and increased photoreceptor survival, confirming in vivo neuroprotection [39].

We are currently creating a zebrafish model of disease, with morpholino and CRISPR/Cas9 gene editing techniques, specific to the *pde6b* p.(Ala733Pro), to utilise as a resource for high-throughput drug screening.

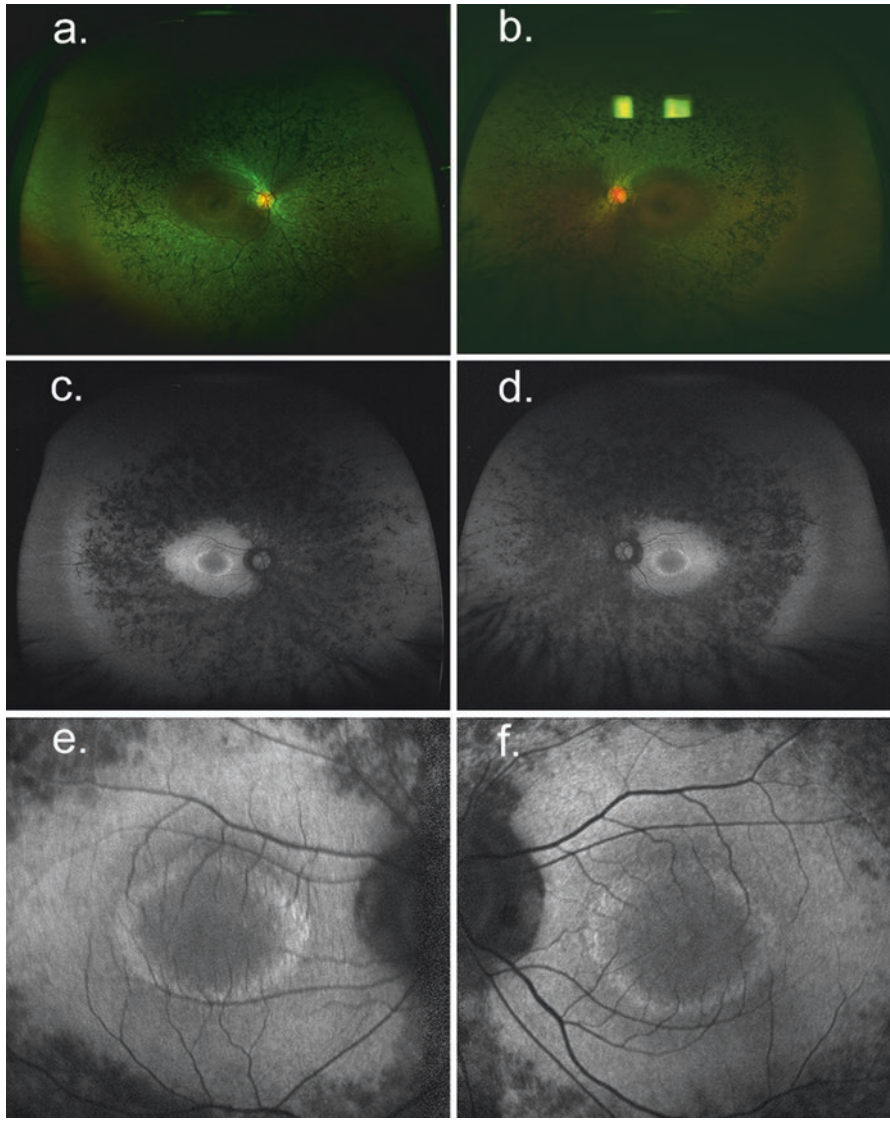

Fig. 5.4 Clinical imaging of the founder *PDE6B* c.2197G>C; p.(Ala733Pro) variant associated with autosomal recessive rod-cone dystrophy. Optos widefield photos OD (**a**) and OS (**b**) demonstrating the characteristic mid-peripheral dense bone spicule pigmentation and retinal atrophy, vessel attenuation, and sparing of the macula in a 45-year-old. Fundus autofluorescence with the Optos OD (**c**), OS (**d**), and Spectralis OD (**e**), OS (**f**), with a perimacular hyperfluorescent ring and bullseye appearance

5.3 X-Linked Disease

5.3.1 Choroideremia

Of the seven probands with a clinical diagnosis of choroideremia and a positive genetic test of the *CHM1* gene, none are Māori or Pacific peoples.

5.3.2 X-Linked Retinoschisis

Of seven probands with X-linked retinoschisis and mutation-positive *RS1* genetic analysis, all are of NZ European ethnicity (Vincent AL et al., IOVS 2017.58:8 ARVO E-Abstract 2770).

5.3.3 X-Linked Congenital Stationary Night Blindness

The clinical features of a large pedigree of Māori ethnicity with incomplete CSNB have been described by Hope et al. [40] and attributable to the previously undescribed *CACNA1F* c.2267T>C, p.Ile756Thr (also known as p.Ile745Thr) allele. All affected males demonstrated severe nonprogressive visual impairment, with congenital nystagmus, altered colour vision, hyperopia, and normal fundi. Some of these males were also intellectually disabled. In this family, the obligate carrier females all showed a moderate to severe ocular phenotype, with decreased vision and congenital nystagmus, and were often highly myopic. In both male and females, the ERG showed a characteristic negative wave form with reduction of both rod and cone responses.

Further individuals from the extended family have subsequently been identified and genotype confirmed, although they did not always directly know of the familial association. A careful history, knowledge of the phenotype, and identification of iwi usually will help target the genetic testing strategy and diagnostic algorithm.

5.3.4 X-Linked Rod-Cone Retinal Dystrophy (XLRP)

We have characterised 19 families with XLRP, attributable to mutations in the *RP2* gene ($n = 1$) and *RPGR*, including ORF15 ($n = 18$) (Vincent AL et al, IOVS 2017.58:8 ARVO E-Abstract 2770). Of these families, three were NZ Māori and one Samoan, and the disease-causing variants observed in this ethnic group were not previously reported and segregated with disease in the family. In *RP2*, the variant c.945_946insT is predicted to cause a premature termination of the protein p.(Asn316*).

The other three variants were in *RPGR*; c.283G>A, p.(Gly95Arg), a missense variant in exon 10, c.1236_1239delAAGA, also resulting in premature termination p.(Glu414GlyfsTer10) and an ORF15 deletion c.2630delA, which segregated with disease in 18 members of the family (#12). A number of obligate carrier females showed significant disease manifestation. In this family, keratoconus also segregated with disease; however the high incidence of keratoconus in this population has previously been discussed (Vincent AL et al, IOVS 2017.58:8 ARVO E-Abstract 2770).

In a further male proband (#11), with three affected daughters, two variants were detected. A heterozygous variant in *C1QTNF5*, c.583dupG, p.(Ala195Glyfs*8), is rare and predicted to be damaging, and he was also hemizygous for *RPGR* c.283G>A p.(Gly95Arg). We were however unable to confirm segregation, the phenotype was not typical for late-onset retinal dystrophy (LORD) described with *C1QTNF5*, and as his sister's son was also reportedly affected, the *RPGR* variant was thought to be the causative allele.

5.4 Maculopathies and Cone/Cone-Rod Dystrophies

5.4.1 *ABCA4*-Associated Disease

In the NZ IRD database, 81 probands have at least 1 pathogenic variant or VUS in *ABCA4* and a clinical picture consistent with disease. Six of these probands identify as Māori, including two sib pairs.

A novel homozygous variant c.5584G>T, p.(Gly1862Cys) observed in one proband (#13) with a maculopathy, occurs in the last nucleotide of exon 39 and is predicted to be probably damaging by PolyPhen-2 with the maximum score of 1.000. This variant does not occur in databases of human variation and was also homozygous in his affected sibling. Both siblings had a similar manifestation of disease.

This variant was not detected initially using the *ABCA4* Asper microarray in 2010, but only subsequently in 2015 with testing on the MVL NGS macular panel, which sequences *ABCA4* in its entirety, including probing for deep intronic variants.

5.4.2 Non-*ABCA4* Maculopathies

At the end of 2016, we identified patients with maculopathies or cone-rod dystrophies (CORD), in which *ABCA4* had been excluded as the cause of disease or a pathogenic variant(s) identified in a gene consistent with the phenotype. Eighty patients were diagnosed with a maculopathy and 22 with a CORD. Vitelliform macular dystrophies (VMD) were the most common maculopathy (25.0%) of which 50.0% were *BEST1* positive. Interestingly none of the individuals with VMD were Māori or Pacific people. The CORDs showed higher proportions of males (68.2%), Māori (27.3%), and Polynesian (9.1%) patients than the maculopathies. A high proportion of Māori and Polynesian patients also lacked genetic characterisation. Eleven patients were identified with *RDS/PRPH2* mutations and exhibited wide phenotypic variability, yet once again none of these individuals were Māori or Pacific peoples, adding further weight to a different genetic spectrum of disease in these ethnic groups.

5.4.3 *CRX-associated* CORD

Patient #14 had a novel heterozygous nonsense variant, c.774 T p.(Tyr258ter), in the *CRX* gene when analysed by the Manchester NGS retinal panel. This change was not present in databases of human variation. This patient had simplex inheritance with a late-onset (age 50) severe cone-rod dystrophy, with vision 6/60 OU at age 55 years. Fundal photography showed paramacular greying and atrophy of retinal pigment epithelium with a bullseye pattern and a central area of loss of photoreceptors on OCT and a central dark area with a hyperfluorescent ring on FAF (Fig. 5.5a).

Electrophysiology was performed to ISCEV standards. Rod-mediated function was identified with reduced photoreceptor A wave amplitude, but relatively normal B wave. Cone-mediated function was identified but significantly reduced in amplitude to about 50% normal and increased in latency for both photopic flash and flicker at 30 Hz. The pattern ERG was poorly defined.

Mutations in the *CRX* gene have been shown to be associated with autosomal dominant LCA type III and as well as AD cone-rod dystrophy and maculopathy [41].

5.4.4 Occult Macular Dystrophy

A 48-year-old NZ Māori male (*Patient #15*) noticed an onset of deterioration in his vision in his late teens and was troubled by glare. His vision measured 6/60 OD and 6/48 OS, with a localised area of photoreceptor abnormality subfoveally (Fig. 5.5b). Full-field ERG was unremarkable, and a pattern ERG showed a reduced p50 wave, consistent with a localised macular abnormality. He was heterozygous for the most commonly reported pathogenic variant in *RP1L1*, c.133C>T. p.(Arg45Trp), as was his affected sister (Fig. 5.5b). Occult macular dystrophy has been reported predominantly in the Japanese population [42].

5.4.5 *BBS9* Cord

A 43-year-old NZ Māori male (*Patient #16*) became aware of shadows in his vision in his early 30s, with vision measuring 6/9 at the time, but deteriorating to 6/60 2 years later and to 1/60 at age 43. Electrophysiology initially showed marked attenuation of cone function and mildly affected rod photoreceptor function. When repeated at age 43, rod function was now barely recordable. The fundus appearance showed widespread sharply demarcated areas of atrophy and small islands of residual retina. The retinal arterioles are mildly attenuated, and both maculae have sub-retinal fibrosis (Fig. 5.5c). The patient had no other systemic features but described multiple relatives on both sides of the family with polydactyly. Testing with the Manchester NGS retinal panel identified compound heterozygosity for two novel variants in *BBS9*: c.205C>A, p.

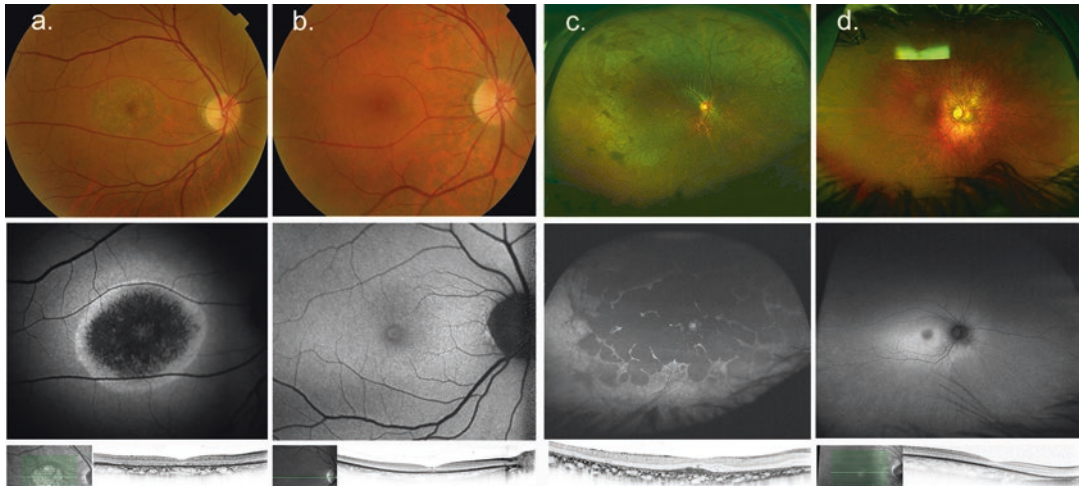

Fig. 5.5 Clinical images of four patients with maculopathies/cone-rod dystrophies. (**a**). *CRX* Patient #14 with a heterozygous *CRX* variant, c.774T p.(Tyr258*): macula photo (*i*), fundus autofluorescence (*ii*) and OCT (*iii*). (**b**). Occult macular dystrophy Optos photo (*i*) OD in Patient #15 with *RP1L1* c.133C>T, p.(Arg45Trp), and fundus autofluorescence (*ii*) and OCT (*iii*) in his affected sister (all OD). (**c**). *BBS9* Patient #16, a compound heterozygote for two novel *BBS9* variants, c.205C>A, p.(Leu69Ile), and c.1014_1015delinsTT p.(Leu338_His339delinsPheTyr) demonstrating sparse pigment clumping and sharply demarcated areas of retinal atrophy (*i*), corresponding to areas of hypoautofluorescence (*ii*), and marked thinning of the outer retina and loss of photoreceptor architecture (*iii*). (**d**). *PROM1* Retinal phenotype of autosomal recessive *PROM1* CORD c.1354_1355insT, p.(Y452Lfs*13), disease (Patient #17), with foveal granularity and peripapillary atrophy (*i*), reduced macular autofluorescence (*ii*), and disturbance of the photoreceptor outer segments in the foveal and perifoveal regions (*iii*)

(Leu69Ile), and c.1014_1015delinsTT p.(Leu338_His339delinsPheTyr), both of which are rare and predicted to be pathogenic. His parents were heterozygotes for a single change each, but other family members were not available for clinical nor genetic characterisation.

5.4.6 *PROM1* CORD

At the age of 3 years, a NZ Māori male was noted to have poor vision and was very light sensitive. Initially a myopic correction was prescribed. When seen at age 7 years, his visual acuity was 6/18 OD, 6/21 OS, with fine granularity noted at each fovea (Fig. 5.5d). Electrophysiology showed reduced rod-mediated amplitudes and non-recordable cone function and pattern ERG. On the NGS panel, a homozygous *PROM1* pathogenic variant c.1354_1355insT, p.(Y452Lfs*13), was present and confirmed heterozygously in his non-consanguineous parents.

5.5 Conclusions

Local knowledge and characterisation of disease prevalence and genetic variation within the New Zealand Māori and Pacific peoples have identified ethnic-specific variation in the spectrum of inherited eye disease compared to the NZ European population. Keratoconus is more frequent but glaucoma less common. Several founder mutations are identified in *ADAMTSL4* associated with autosomal recessive ectopia lentis and *PDE6B* in ARRP. Knowledge of these changes, the presenting phenotype, and the regional origin and iwi of the patient can simplify the diagnostic algorithm, to inform targeted genetic testing. As this population is not well represented in databases of human variation, segregation and in silico analysis are paramount to determining pathogenicity of any variant detected.

Although many of our probands have not undergone state-of-the-art NGS gene panel inves-

tigation, the unique and novel variants identified to date provide a strong argument to justify further investigation within the NZ Māori and Pacific peoples and a high likelihood of identification of further novel genetic variation as the cause for their eye disease.

Informed Consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

Additional informed consent was obtained from all patients for which identifying information is included in this article.

No animal studies were carried out by the authors for this article.

Compliance with Ethical Requirements Author ALV has no conflicts of interest to declare.

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Genetics of Ocular Diseases in Malaysia

6

A. T. Liza-Sharmini and T. A. Kamalden

Abstract

Understanding the role of genetics in clinical practice has evolved drastically in Malaysia. However, the understanding of genetics in ocular diseases is still at the infancy. In this chapter, we summarize the publications on genetic studies involving Malaysian population on glaucoma, age-related macular degeneration, diabetic retinopathy, retinopathy of prematurity, and retinoblastoma. Potential susceptibility genetic markers for primary open-angle glaucoma (POAG) and primary angle-closure glaucoma (PACG) were identified through single gene analysis and genome-wide association study (GWAS). TGF-ß signaling pathway is the potential susceptibility gene for POAG. MYOC gene was also identified in a large Malay family with juvenile-onset open-angle glaucoma (JOAG). Single nucleotide polymorphism (SNP) of VEGF+405 may play a role in wet age-related macular degeneration in Malaysian population, while SNP of 2245G/A was found to be associated with diabetic retinopathy, possibly through NF-κβ-mediated pro-inflammatory pathway. Exome-wide association studies (EWAS) on a pilot cohort of 20 premature Malaysian infants, different loci of SNPs in LRP5, FZD4, ZNF408 (chromosome 10), and KIF 11 (chromosome 11) genes were identified. No specific hot spot in RB1 gene in Malaysian children with retinoblastoma was found. In general, more exploration is needed in understanding the genetic influences in ocular diseases in Malaysia.

Keywords

Ocular genetics · Malaysia · Glaucoma · Retinopathies

6.1 Introduction

Genetic and molecular research in Malaysia is still at its infancy. The Malaysia Institute for Medical Research (IMR) was established in 1900, but the cytogenetic service was only available in 1968 [1]. About 28 years later, molecular diagnostic service was established in 1996. Currently, there are many genetics laboratories in Malaysia that cater for research rather than diagnostic services. However, the research activities have not been coordinated

A. T. Liza-Sharmini (✉)
Department of Ophthalmology, School of Medical Sciences, Universiti Sains Malaysia, Kota Bharu, Kelantan, Malaysia
e-mail: liza@usm.my

T. A. Kamalden
University of Liverpool, Liverpool, UK

Faculty of Medicine, Department of Ophthalmology, UM Eye Research Centre, University of Malaya, Kuala Lumpur, Malaysia

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resulting in many laboratories conducting similar tests and being counterproductive. Till now, there is no laboratory in Malaysia that caters for diagnostic service for ocular diseases.

Genetic studies on ocular diseases were almost nonexistent until about 20 years ago. This is due to the emphasis given to other aspects such as in the development of ophthalmic services, training of ophthalmologists and supporting staffs, and strategic planning for prevention of common causes of blindness such as cataract. However, with the increasing number of ophthalmologists and availability of training in molecular research among clinicians, the interest in ocular genetic has escalated.

Malaysia is a multiracial country bordered by Thailand and Indonesia. Malays, Chinese, and Indians are the main ethnic groups in Malaysia. Malays and other indigenous people contributed to 67.4%, Chinese 24.6%, Indian 7.3%, and others 0.7% [2]. This diversity has led to variation in phenotype and genotype of ocular diseases. Most ocular diseases are complex, and understanding the disease in a multiracial country like Malaysia is challenging.

Currently, knowledge on clinical presentation and genetic predisposition on ocular disease in Chinese and Indians is abundant [3, 4]. This is basically due to advancement of research from China and India. Due to genetic inheritance, the findings can be extrapolated to Chinese and Indians residing in Malaysia with the assumption of minimal environmental effect on these populations. There is minimal knowledge on ocular genetics in Malays and other indigenous people in Malaysia. Malays contribute to 5% of general population in the world [5]. In this chapter, we concentrate on the available genetic data of common ocular diseases such as glaucoma, age-related macular degeneration (ARMD), retinopathy of immaturity (ROP), and retinoblastoma (RB) among the Malaysian population.

6.2 Glaucoma and Genetics in Malaysia

Glaucoma is a chronic progressive optic neuropathy with special characteristics of structural and functional damage. It is one of the major causes of irreversible blindness in Malaysia. Based on the Malaysia National Eye Survey II, glaucoma contributed 7% of blindness and 3% of severe visual impairment [6]. Glaucoma is a complex disease with interplay between genetic predisposition and environmental factors [7]. Formulating the diagnosis of glaucoma is challenging without consensus in structural and functional changes, intraocular pressure (IOP), and other factors. For years, various definitions were adopted resulting in difficulty in providing an evidence-based management. However, since the formation of an expert panel in glaucoma, World Glaucoma Association in 2001, a series on consensus have been developed to standardize diagnosis and patients' care.

Glaucoma has all the characteristics of a complex trait including locus heterogeneity, polygenic inheritance, phenocopies, and incomplete penetrance [8, 9]. To complicate the matter further, the types of glaucoma are usually determined by various mechanisms that end up with a similar final pathway, glaucomatous optic neuropathy. Furthermore, the exact pathogenesis of glaucoma is not well understood. Without a doubt, genetic component has a role in glaucoma. First-degree relatives of primary open-angle glaucoma (POAG) patients have a tenfold higher risk of developing POAG compared to general populations [10]. Family history is one of the risk factors for glaucoma particularly POAG, primary angle-closure glaucoma (PACG), and juvenile-onset open-angle glaucoma (JOAG) [11, 12].

6.3 Single Gene Analysis

Identification of genetic association to glaucoma begins with single gene analysis. Thus, so far, there are various potential genetic loci which

have been identified as susceptibility gene of glaucoma. Among these genetic loci, only three candidate genes were identified: myocilin gene (MYOC), optineurin gene (OPTN), and WD40-repeat 36 gene (WDR36) [13–15]. There is no available genetic screening for OPTN and WDR36 in Malaysian population.

6.3.1 Myocilin Gene (MYOC)

MYOC consists of 3 exons separated by 2 introns and a 5 kb promoter region, encoding for 55–57 kDa myocilin protein with 504 amino acids and an isoelectric point approximately 5.21 [16]. It encodes a secretory trabecular meshwork-inducible glucocorticoid response (TIGR) myocilin protein, which is expressed in many human tissues including the iris, ciliary body, and trabecular meshwork [17]. TIGR myocilin protein has an amino terminal signal sequence, an olfactomedin domain, a myosin-like domain, and a leucine zipper domain [18]. The majority of genetic variations were found in exon 3 which encodes olfactomedin-like domain [19]. In spite of vast research on MYOC in many types of glaucoma, the impact of MYOC remains for POAG and JOAG. However, MYOC mutations were only found in 2–4% of POAG patients and 8–30% in JOAG patients [19, 20].

As yet, there is no available data on the association between MYOC and POAG among Malaysians. A screening of MYOC among a large Malay family with JOAG was conducted and 122 of the family members were thoroughly examined [21]. A total of 32 probands were identified: 11 new cases and 22 known cases. Autosomal dominant inheritance pattern with variable penetrance was observed in this family (Fig. 6.1). Penetrance in JOAG is age dependent and mutation specific [19]. Disease-causing mutations (DCM), Asn480Lys (C→A at position 1440 in exon 3) and a synonymous polymorphisms IVS2 730 +35G>A (rs2032555), were also detected in this family. Asn480Lys was found in all probands with JOAG except for two [21]. Additionally, six probands with Asn480Lys but without the disease development were identi-

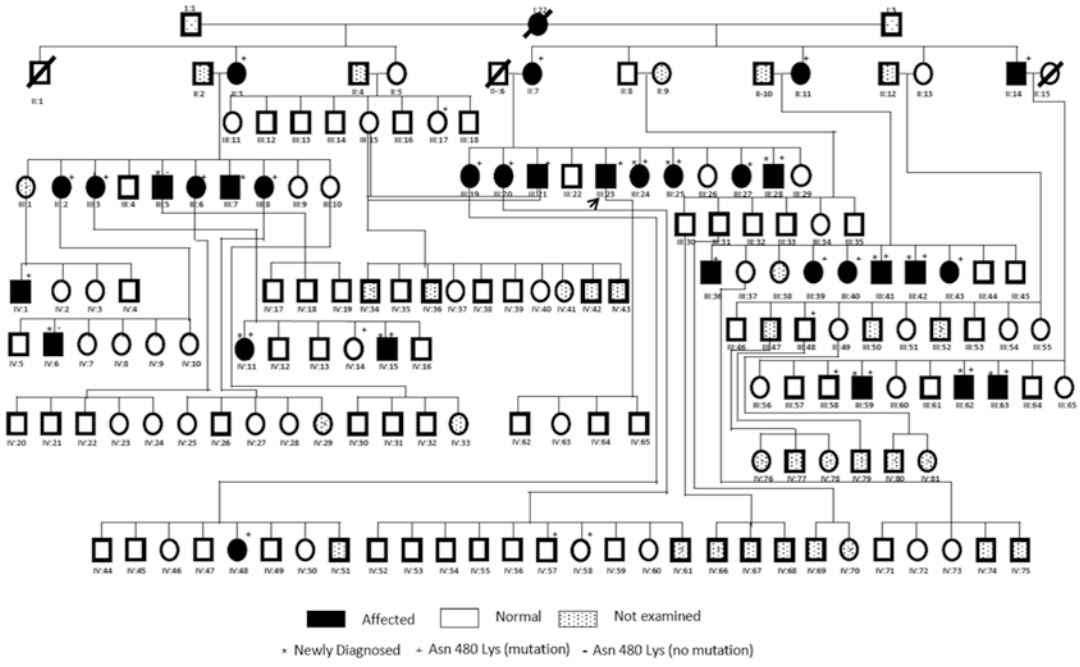

Fig. 6.1 Pedigree chart of a large Malay family with juvenile-onset open-angle glaucoma and distribution of Asn480Lys of MYOC

fied. Other research has shown that Asn480Lys was also identified in European population and southern Indian POAG patients [22, 23]. Similarly, non-synonymous polymorphism IVS2 730 +35G>A was found in Chinese and southern Indian patients with POAG [24, 25].

Lys480Lys and IVS2 730 +35G>A were susceptible markers for Malay patients with JOAG with linkage disequilibrium of 0.619 [21]. Probands with Lys480Lys were detected at mid-twenties with lower IOP at diagnosis, while IVS2 730 +35G>A was detected in probands at early twenties but with higher IOP at diagnosis. There was no association of this DCM and non-synonymous polymorphism with severity of JOAG. Based on linkage disequilibrium (0.619), there is a possibility of combination effect of Lys480Lys and IVS2 730 +35G>A. However, this potential effect was not investigated in this study.

6.3.2 Beta-2 Adrenoreceptor Gene (ADRB2)

Most of pressure-lowering drugs work through interaction with receptors found in the trabecular meshwork, uveoscleral pathway, and ciliary body. Beta adrenoreceptor (ADRB) is believed to play an important role in aqueous humor production and outflow, regulating IOP [26]. ADRB is divided into three major subtypes: beta-1 (ADRB1), beta-2 (ADRB2), and beta-3 (ADRB3) [27]. Their expression differs according to the organ. For example, ADRB2 is predominantly found in the human iris, ciliary body, and blood vessels to optic nerve head [26]. Polymorphism and mutation of beta-2 adrenoreceptor (ADRB2) gene may alter the aqueous production and potentially play an important role in pathogenesis of glaucoma. ADRB2 gene is an intronless gene located at chromosome 5q31-32 [28].

ADRB2 gene was screened using multiplex polymerase chain reaction (PCR) for polymorphisms at beta upstream peptide -47T>C (rs1042711), intronic polymorphism -20T>C (rs1801704) and exonic polymorphisms 46A>G (rs1042713), and 79C>G (rs1042714) and 491C>T (rs1800885) on 97 glaucoma patients (POAG and NTG) and 100 controls [29]. Out of these 197 subjects, there were 123 Malay and 74 Chinese subjects who resided in Malaysia. Based on univariate analysis and without considering the potential effect of racial stratification, there was no significant association between five polymorphisms of ADRB2 and susceptibility to POAG and NTG. However, using stratified Mantel-Haenszel meta-analysis, minor allele 79C>G (79G) reduced the risk of POAG by 0.3-fold (95% CI 0.1, 0.7) and -20T>C (-20T) increased the risk of NTG by 2.0-fold (95% CI 1.1, 3.7).

6.3.3 Prostanoid Receptor Gene (PTGFR)

Currently, prostaglandin analog is a potent first-line topical drug for treatment of glaucoma. It has gained popularity over the past 20 years since its first introduction in 1998 [30]. Prostaglandin analog acts on specific receptor: prostanoid (FP) receptor. Human FP receptor is encoded by $PTG_{2\alpha}$ gene known as PTGFR. PTGFR is located at short arm of chromosome 1(13.1) [31]. It consists of 4 exons and 3 introns spanning 43.3kb [32]. The first exon is relatively short (165 bp) and comprises most of the 5′-untranslated region (5′-UTR). Intron 1 is approximately 1.3 kb in size and may contain part of the promoter region. The second exon (870 bp) consists of approximately 70 bp of untranslated region and encodes the remaining 5′-UTR, and the rest of the second exon is then translated. The translated region continues up to Leu266 near the end of transmembrane VI. However, it is interrupted by the large second intron (4.3 kb) and the third intron (38.5 kb). A small third exon is approximately 70 bp in size. The fourth exon is quite large, spanning 3344 bp, but only a small fragment is translated, and the rest is the 3′-untranslated region (3′-UTR).

Screening of PTGFR gene was conducted on 90 glaucoma patients and 90 control subjects. Among these patients and subjects, there were 124 Malays and 56 Chinese patients. A total of 63

single nucleotide polymorphisms (SNPs) were identified with 1 SNP found in the exon, and the rest were intronic polymorphisms [29]. The majority of the intronic polymorphisms (43 SNPs) were found near the intron 3. Exonic SNP rs3766331 was found within exon 4 [33]. A novel SNP rs3766332 was found at the flanking region of exon 4 [34] (Hoh et al., 2007). Microsatellite instability (MSI) was also found in intron 3, "CA" deletion and "TA" insertion [29].

Among the intronic SNPs, three SNPs were found as potential susceptible markers for glaucoma in Malaysian population: rs2146489, rs11162505, and rs556817. Non-synonymous polymorphism of rs2146489 was found to increase the predisposition to glaucoma 3.1 fold (95%CI 1.1, 8.8) in Malaysian population. SNPs rs11162505 and rs556817, which are located several base pairs apart, conferred protective effect against glaucoma susceptibility in Malaysian population. There was no evidence of linkage disequilibrium between these two intronic SNPs, but it was shown that rs11162505 was in linkage disequilibrium with rs554185. There was significant association of GG ($p = 2.0 \times 10^{-4}$) and GA ($p = 3.0 \times 10^{-4}$) haplotypes of rs11162505 and rs554185, respectively, with susceptibility to glaucoma.

PTGFR gene was also screened in Malay and Chinese patients with primary angle-closure (PAC) patients residing in Kelantan, a northeastern state in peninsular of Malaysia. Kodisvary et al. [35] evaluated the potential association between ocular biometry in PAC patients and PTGFR polymorphisms. Ocular biometry is considered as one endophenotype for PACG. The potential role of endophenotypes of glaucoma further complicates the complexity of phenotype in glaucoma. Endophenotypes are plausible quantitative traits or risk factors with strong genetic components that are related but not part of the symptom of the glaucoma [36]. These include IOP, central corneal thickness (CCT), vertical cup to disc ratio (VCDR), cup and disc area, and ocular biometry for angle-closure glaucoma. There was high heritability of endophenotype of POAG; 0.55 for IOP, 0.48–0.66 for VCDR, 0.75 for cup area, and 0.72 for disc area [37, 38]. However, there is no available data for Malaysian population.

Kodisvary et al. conducted a cross-sectional study to find the association between PTGFR and acute angle-closure presentation (APAC) in PAC patients [35]. Her team attempted to answer what triggers APAC in a susceptible individual. Why certain susceptible individuals remain asymptomatic and insidiously developed optic neuropathy? There is a possibility of the genetics makeup that is responsible in determination of ocular biometry and inducing acute attack [39]. Shallower anterior chamber depth (ACD) and smaller anterior segment dimension increased the susceptibility to PACG [40, 41].

A total of 27 PAC patients and 30 age-matched controls were involved in this study. Out of 27 patients with PAC, 16 presented with history of APAC [35]. Genetic screening for rs3766332 A>T and rs3766331 A>G of PTGFR was conducted using denaturing high-performance chromatography (dHPLC) technique. Samples with heteroduplex peak were then subjected to Sanger sequencing technique. There was a significant association between rs3766332TT and APAC among PAC patients [35]. Ocular biometry was also evaluated in this study using A-scan ultrasonography, anterior chamber depth (ACD), lens thickness, and axial length. However, there was no significant association of these two SNPs with ocular biometry of PAC patients in this small study. The FP receptor plays an important role in a cascade of inflammatory reaction; there is element of inflammation in APAC. rs3766332TT is a potential candidate genetic marker for APAC.

A study conducted in Taiwanese patients with APAC identified rs2664538 of MMP-9 gene as potential candidate markers [42]. Matrix metalloproteinase (MMP) plays a role in regulation of cytoskeletal changes of the uveoscleral outflow [43]. Binding of prostaglandin analog to FP receptor that is regulated by PTGFR gene is known to induce remodeling of extracellular matrix (ECM) of the sclera at the uveoscleral and trabecular meshwork [44]. Widening of collagen in uveoscleral pathway facilitates the outflow of the aqueous and reduction of the intraocular pressure. There is a possibility of gene-gene interac-

Table 6.1 Summary of single gene screening conducted on glaucoma patients and control subjects in Malaysia

Gene	Type of glaucoma	Race	Findings
MYOC	JOAG	Malays	Asn480Lys
			IVS2 730 +35G>A
ADRB2	POAG	Malays	79C>G (79GG)
	NTG	Chinese	-20T>C (-20T)
PTGFR	POAG	Malays	rs2146489
	NTG	Chinese	rs11162505
			rs556817
PTGFR	PACG	Malays	rs3766332
		Chinese	rs3766331

tion of PTGFR and MMP genes. However, the sample size is rather small with high potential of selection bias. A larger sample size is definitely necessary to confirm this finding. A replication study is also important.

Based on the available single gene analysis in the Malaysian population, there is a potential role of PTGFR in susceptibility of POAG and NTG. For intronic polymorphism of PTGFR, rs3766332 is also a potential marker for APAC. The ADRB2 gene may also play a role in susceptibility to open-angle glaucoma. However, the sample size was rather small for the studies involving screening of PTGFR and ADRB2 in Malaysian population. In addition, only Malays and Chinese were involved. MYOC mutation is found to play a strong association in a Malay family with JOAG (Table 6.1).

6.4 Genome-Wide Association Studies

With advancement of technology in genetics, screening of entire human genes is currently possible in a shorter time and at an acceptable cost. To maximize the outcome, collaborative work with various institutions involving multiple populations has been initiated. A group of investigators in Malaysia were involved in this smart partnership under the leadership of investigators in Singapore Eye Research Institute (SERI), Singapore.

Malay PACG patients residing in Malaysia were included in stage 1 genome-wide association study (GWAS) involving five countries. Based on the findings of stage 1 and stage 2 (replication study of six countries), three susceptible genetic markers of PACG were identified: rs11024102 in PLEKHA7, rs3753841 in COL11A1, and rs1015213 located at intergenic region between PCMTD1 and ST18 [45] . In this study, a total 1917 PACG patients and 8943 control subjects were involved. PLEKHA7 encodes pleckstrin homology domain containing protein 7 which is involved in maintenance stability of adherence junctions [46]. Adherence junctions are abundant in the eye that regulates paracellular permeability [47]. COL11A1 encodes one of the alpha-chains of type XI collagen. Mutation of COL11A1 is responsible for ocular, orofacial, auditory, and skeletal manifestation of Marshall syndrome, Stickler syndrome, and Stickler-like syndrome [48]. Variants in COL11A1 are believed to play a role in fibrillar collagen matrix formation resulting in shorter eyeball and overcrowding of the anterior chamber. In addition, COL11A1 was also expressed in human trabecular meshwork, which may play a role in the aqueous outflow in PACG patient [49]. The potential role of intergenic SNP rs1015213 is still unknown.

Currently, another five susceptible markers for PACG were identified in discovery stage of GWAS study: rs3816415 in EPDR1, rs736893 in GLIS3, intergenic SNP rs3739821 in between DPM2 and FAM102A, rs1258267 in CHAT, and rs7494379 in FERMT2 [50]. In this study, a total of 10,503 PACG patients and 29,567 control subjects from 24 countries were involved. Four centers in Malaysia were involved in this discovery stage and the data collection includes all races in

Malaysia. The mRNA expression of these markers on human ocular tissues including on the iris, ciliary body, trabecular meshwork, cornea, lens, retina, choroid, and optic nerve head was conducted using RT-PCR technique. Protein expression of these markers on ocular tissue was also identified. In general, protein expression corroborated well with mRNA expression except for GLIS3 [50].

EPDR1 encodes glycosylated type II transmembrane protein known as ependymin-related 1 [51]. FERMT2 encodes protein called pleckstrin homology domain containing family C member 1 (PLEKHC1), a component of extracellular matrix [52]. Both EPDR1 and FERMT2 have a role in cell adhesion [51, 52]. This further supports the role PLEKHA7 in cell adherence, whereby the knowledge was obtained from the earlier stage of GWAS [45]. CHAT encodes choline acetyltransferase, an enzyme responsible for synthesis of acetylcholine neurotransmitter which is important in pupillary constriction [53]. Pupillary dilatation may cause pupillary block and APAC in susceptible individual. There is potential genetic predisposition through the variation of CHAT which increases the risk of PACG. However, the role of intergenic SNP rs3739821 and GLIS3 in susceptibility to PACG is still not clear [54].

ACD has shown high heritability based on Guangzhou Twin Study [55]. ACD is considered as endophenotype for PACG. In another GWAS study, rs1401999 in ABCC5 was identified as quantitative trait loci for ACD in the stage 1 involving 5308 participants [56]. ACD was measured using IOL master, and only the right eye measurement was obtained from Singapore Malay Eye Study (SiMES), Singapore Indian Eye Study (SINDI), and Beijing Eye Study (BES). Based on meta-analysis, the effect size was −0.045 mm ACD for per minor allele of rs1401999 ($p = 8.17 \times 10^{-9}$). Stage 2 involved case-control study on 2422 PACG patients and 9193 controls from seven countries using SNP array analysis. Malay patients residing in Malaysia were included in this case-control study. There was modest association between minor allele (C) of rs1401999 and PACG with per allele odds ratio of 1.13 (95% confidence interval 1.06–1.22, $p = 0.00046$). ABCC5 is also found to be expressed in human ocular tissue including the iris, ciliary body, and lens [57].

A two-stage exome chip study involving discovery and replication stage was conducted in POAG patients and control patients [55]. Malay patients with POAG residing in Malaysia were included in the replication stage of this GWAS. CDKN2B-AS1 rs2157719 was identified as potential genetic marker in both stages with per allele effect of 0.71 ($p_{meta} = 2.81 \times 10^{-33}$) involving 12,677 POAG patients and 36,526 controls. CDC-TGFBR3 rs1192415 (OR 1.13, $p_{meta} = 1.6 \times 10^{-8}$) and FNDC36 rs4894796 (OR 0.93, $p = 1.40 \times 10^{-5}$) were identified in the replication stage involving 9133 POAG patients and 26,780 controls. All three loci, CDKN2B-AS1, CDC-TGFBR3, and FNDC36, contain genes which may contribute to transforming growth factor-β (TGF-β) signaling. TGF-β is postulated responsible for retinal ganglion cell death and glaucomatous optic nerve damage. TGF-β signaling plays important in various diseases including cataract and glaucoma.

Based on available GWAS in Malaysian population, susceptibility markers for PACG seem to associate with gene controlling cell adherence junction and collagen formation, suggesting a close connection with changes in anterior chamber structure and aqueous formation and outflow, while susceptibility markers for POAG are potentially related to TGFBR pathway. Currently, there is a group of researchers searching for susceptibility genetic markers for progression of POAG and PACG in Malays.

6.5 Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a major cause of irreversible blindness in the world. AMD is a progressive disease which affects persons aged 50 and above. Genetic predisposition was first shown to have strong association in AMD based on the landmark genome-wide association study (GWAS) by Klein in 2005 [57].

This was followed by several GWAS from different population around the world. Among the most influential genes associated with AMD are complement factor H (CFH) [58, 59] and age-related maculopathy susceptibility 2(ARMS2) and HtrA serine peptidase 1 (HTRA1) [60–62].

More recently, a GWAS and exome-wide (EWAS) association studies have reported the genotypes of exudative AMD among East Asians [63]. In the study, in addition to three novel loci of mutation, an uncommon mutation at CETP (D442G) was found to be specific to East Asian cohort of exudative AMD patients. In a study of 135 patients with age-related macular degeneration in Malaysia [64], VEGF+405 polymorphism was found to be associated with the wet type of AMD. This association was also found in Tunisian patients with AMD [65]. Several studies are currently underway to determine the genotype of AMD in the Malaysian population.

6.6 Diabetic Retinopathy

Diabetes mellitus (DM) is a major cause of morbidity and mortality in the world and is fast becoming a global pandemic. In 2014, it is estimated that 387 million people have diabetes worldwide, and this number will increase to 592 million by the year 2035. The current national prevalence of DM in Malaysia is 16.6%, with an estimated 3.2million people with DM, compared to 1.9 million in 2013 [66]. Diabetic retinopathy is the third common cause of avoidable blindness and was responsible for blindness in an estimated 5 million of the world population in 2002 by the World Health Organization [67]. In Malaysia, DR is the leading cause of visual loss in the working age group. The prevalence of diabetic retinopathy in Malaysia is estimated at 36.8% [68], which is similar to worldwide prevalence of 37% [69].

The role of genetic susceptibility in DM has been well known. High concordance between twins with DR has been reported previously [70, 71]. Most genetic association studies in DR were performed using candidate gene approach. One of the genes implicated in DR is the gene for receptor of advanced glycation end product (RAGE) [72, 73]. In the Malaysian cohort, gene polymorphism 2245G/A was found to be associated with DR among Malaysian patients possibly through NF-κβ-mediated pro-inflammatory pathway [74, 75]. Conversely, Gly82Ser, 1704G/T, 2184A/G, -429T/C, and -374T/A gene polymorphisms were reported to have a lack of association with DR [76, 77].

6.7 Retinoblastoma

Retinoblastoma is the commonest primary intraocular tumor in children less than 5 years old [78]. About 14.5 new cases of retinoblastoma are diagnosed yearly in Malaysia [79]. Several studies have reported retinoblastoma gene mutations in the Malaysian population [80–82]. In a meta-analysis of 932 reported RB1 mutations, Valverde et al. showed heterogenous distribution of mutations across the RB1 gene from around the world [83]. Mohd Khairul et al. showed similar distribution of retinoblastoma mutation in Malaysia [84]. The SNP 153104 in RB1 intron 18 was reported to be one of the common SNPs found in Malaysian children with retinoblastoma [80]. There is no "hot spot" of RB1 gene found in Malaysian children with retinoblastoma [80–82, 84]. In fact, there is no mutation or SNP found in N and C-termini and pocket A and B domain of RB1 in a number of children with sporadic RB in Malaysia [80–82]. There is possibility of variations at the intronic and promoter region of RB1 which is not screened in Malaysian population.

6.8 Retinopathy of Prematurity

Retinopathy of prematurity (ROP) is a visually threatening disease affecting premature infants' retina caused by abnormal retinal angiogenesis. Known major risk factors associated with ROP include low gestational age, low birth weight, and high supplemental oxygen [85, 86]. The role of genetic influence in ROP has been proposed [87] based on similarities in ROP clinical phenotypes with other genetic conditions such as Norrie

disease and familial exudative vitreretinopathy. Genetic variants may also explain observed susceptibility to developing advanced ROP in certain groups of premature infants. Genes which have been linked to possible association with ROP include *NDP* [88, 89], *FZD4* [90, 91], *LRP5* [92, 93], and growth factors genes such as *VEGF* [94–96] and *BDNF* [97, 98]. The influence these genes exert on angiogenesis pathways is believed to have an effect on ROP development and progression [99].

In a recent exome-wide association studies (EWAS) on a pilot cohort of 20 premature Malaysian infants consisting of 10 infants with ROP and 10 without, we found different loci of single nucleotide polymorphisms in LRP5, FZD4, ZNF408 (chromosome 10), and KIF 11 (chromosome 11) genes (unpublished data). Further validation studies are required to determine the genetic associations in the Malaysian population.

Informed Consent All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

Animal Studies No animal or human studies were carried out by the authors for this article.

Compliance with Ethical Requirements Author Liza-Sharmini AT has received speaker honorarium by Pfizer Malaysia Sdn Bhd, Novartis Malaysia, and Santen Malaysia. She was involved in clinical trial study sponsored by Allergan.

Author Tengku Ain Kamalden declares that she has no conflict of interest.

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7 Challenges and Opportunities in Genetic Research from the Perspective of a Tertiary Eye Care Hospital in Bangladesh

Nazmun Nahar, Mohammad Ibn Abdul Malek, and Bipul Kumer De Sarker

Abstract

Clinical programs in developing countries like Bangladesh are focused more on common conditions and less toward advanced research. But the potential for molecular research is considerable, with heritable factors linked in both common and uncommon diseases such as primary open-angle glaucoma, primary angle-closure glaucoma, diabetic retinopathy, vasculitis retinae and age-related macular degeneration, congenital glaucoma, and retinitis pigmentosa, among others. Less common ocular diseases also lend themselves to diagnosis where all investigations are not always available. This paper considers the epidemiology of some common and uncommon ocular conditions in glaucoma and retina at a tertiary referral center in Bangladesh, to present the magnitude of ocular disease as well as outline the huge potential of genetic research. As the technology for genetic analysis already being available, emphasis should be placed on its use in ophthalmology, so that new therapeutic modalities can be developed for certain diseases and to provide treatment for people who at present have no recourse for either diagnosis or treatment.

Keywords

Molecular research · Epidemiology · Retina · Glaucoma

7.1 Introduction

Bangladesh is a small country, albeit with a huge populace (over 160 million and counting) [1]. The Ispahani Islamia Eye Institute and Hospital is a tertiary referral center, with around 2000 patients being seen daily. Due to the huge burden of patients and the necessity of clinical management of these patients, research thus far has taken a backseat. Nationwide statistics are not reliably available for most ocular diseases. Clinical programs in the background of constrained funding mean treatment has been limited to mostly cataract and more recently the emerging ocular diseases outlined in Vision 2020. Molecular research in ophthalmology is at present negligible. While there is biomedical research going on in Bangladesh, ophthalmology as a whole has not yet taken the step into the genetics of the origin and progression of ocular diseases. With this paper, we seek to demonstrate the magnitude of disease in our country from the perspective of the retina and glaucoma departments of a tertiary referral center and the scope for research into the genetics of such diseases.

N. Nahar (✉) · M. I. Abdul Malek · B. K. De Sarker
Ispahani Islamia Eye Institute and Hospital,
Dhaka, Bangladesh
e-mail: Nazmun.nahar.retina@islamia.org.bd; Ibn.malek@islamia.org.bd; Bipul.sarker@islmaia.org.nd

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7.2 Genetics

It is known that a wide variety of retinal diseases have their origins in the genetic pattern passed down through successive generations. The use of genetics to predict the course of disease and to determine the risk and prognosis of certain diseases will no doubt be invaluable in the future.

In the field of retina, diabetic retinopathy (DR) is one of the major diseases, and heritable factors have been implicated in 25–50% of patients who develop diabetic retinopathy, and genetic factors are known to influence the severity of diabetic retinopathy [2]. Certain genes, including ALR2, VEGF, and RAGE genes, have been implicated in DR, but a consistent association has not been proven in all populations [2]. Age-related macular degeneration(ARMD), known as the leading cause of blindness in a Western population, has a complex multifactorial etiology, but a major risk variant within the complement factor H gene (CFH) has been identified [3]. Idiopathic polypoidal choroidal vasculopathy is closely associated with ARMD, with genes in *LOC387715* rs10490924 associated with PCV and its clinical manifestations. *HTRA1*, *CFH*, and *C2* were also found to be associated with PCV [4]. Retinitis pigmentosa (RP) is a well-documented cause of progressive visual loss, which has extensive research into the underlying genetics [5]. Although RP is said to have no ethnic predilection, the pathogenesis is thought to differ between populations, and numerous genes have been identified with nonsyndromic autosomal dominant, autosomal recessive, and X-linked RP [5]. Vasculitis retinae comprise a rare group of disorders that has been associated with numerous HLA alleles, associated with many autoimmune disease [6]. From Bangladesh Perspective, tuberculosis is one of the most common causes of retinal vasculitis; nevertheless, a large proportion of patients are idiopathic [7].

Primary open-angle glaucoma (POAG) and primary angle-closure glaucoma (PACG) comprise the majority of patients presenting to a glaucoma dept. More than 30 chromosomal loci and 4 genes (MYOC, OPTN, NTF4, and WDR36) have been linked to POAG, whereas PACG has been associated with PLEKHA7 and COL11A1 genes. In congenital glaucoma, there are mutations in two genes causing autosomal recessive congenital glaucoma, CYP1B1 coding for cytochrome P450 1B1 and LTBP2. Recently, a genome-wide association study has identified a locus on 15q24, and the lysyl oxidase-like 1 (LOXL1) gene has been strongly associated with both pseudoexfoliation syndrome and pseudoexfoliative glaucoma. Glaucoma is associated with anterior segment dysgenesis, Axenfeld-Rieger syndrome, and aniridia caused by mutations in PITX2, PAX6, and FOXC1, and mutations in these genes cause dominantly inherited disease. In normal-tension glaucoma (NTG), there is a duplication of the TBK1 gene that interacts with optineurin, a protein that is also a rare cause of NTG.

7.3 Epidemiology and Research Opportunities

There is paucity of data on epidemiological studies of eye diseases in Bangladesh. Therefore, we have considered the patients seen at Islamia over the past year. Although not fully representative of population patterns, an assumption about the magnitude of disease can be made from this data. Over the year spanning July 2016 to June 2017, 282,712 patients were seen at the Ispahani Islamia Eye Institute and Hospital. Of these, a total of 47,145 patients (17%) were seen in the vitreo-retina dept, with 23,406 patients (8%) being seen by the glaucoma department.

Primary angle-closure glaucoma (PACG) constitutes 43.1% (10,095) of all patients, a statistic surprising as POAG is more common in neighboring India [8, 9]. This merits further research into the genetic roots of this disease in a Bangladeshi population. Primary open-angle glaucoma constitutes 25.5% (5966), juvenile open-angle glaucoma (JOAG) 1.4% (335), normal-tension glaucoma (NTG) 2.4% (549), pseudoexfoliation 0.3% (69), and congenital glaucoma 1.06% (248). The remainder are secondary glaucomas (26.2%), with an associated secondary cause. Most of the patients had unilateral blindness at the time of presentation,

emphasizing the need for a tool to screen those at potential risk. Since most of these diseases have genetic associations, a detailed analysis of their gene patterns would render invaluable information regarding the likelihood of glaucoma and the factors affecting its progression. These numbers are just the tip of the iceberg of the true situation in the community as many patients remained undiagnosed at community level.

Over a 1-year period from July 2016 to June 2017, a large percentage (21%, number 9991) of patients were DR, of which 42% (4296) was proliferative and nearly equal numbers of non- proliferative (30%, 3000) and advanced diabetic eye disease (27%, 2695). The higher proportion of severe cases indicates the pattern of a tertiary referral center, where the worse cases are usually referred. These patients can provide a rich pool into the genetic analysis of the causative factors of progression and additionally will give an insight into the genetic underpinnings of DR in a Bangladeshi population. ARMD, as already mentioned, is a leading cause of blindness in a Western population. In our retina department, they comprised 4% (1785) of all patients, which may indicate a decreased tendency to present or a tendency to present only when the disease is advanced. The number was approximately split between the dry and wet varieties, with IPCV making up a good number of these cases. The genetic variabilities relating to the progression of ARMD have been researched, but this represents a chance for the genetics of progression in a Bangladeshi population, so that the findings can be compared and contrasted with those in the Western world, where most of ARMD research till date has focused. Retinitis pigmentosa has been extensively researched, with numerous different alleles implicated, and comprises approximately 2.5% of patients of our outpatients. A focus on the genetics and phenotypic expression of these diseases in a Bangladeshi population, and the expression of both syndromic and non-syndromic RP in these patients, is a potential area for investigation. Vasculitis retinae, comprising around 2% of our patients, is an idiopathic disorder that commonly affects a South Asian population, and the genetic component of these apparently idiopathic cases is an absolute necessity, so as to identify cases at risk and develop future therapies. Macular dystrophies are frequently diagnosed, but subclassification requires accompanying electroretinogram/electroculogram, which at present is not available. A genetic mapping of these patients would identify the specific underlying genetic defect and would aid in the development of future gene therapy. Additionally, many rare diseases including familial exudative vitreoretinopathy, Coats' disease, and intermediate and posterior uveitis including Vogt-Koyanagi-Harada (VKH) syndrome have a frequent presentation and have a genetic basis that requires further investigation.

7.4 Challenges

Resources have been the main challenge till now, since the volume of patients have meant that patients who can be given immediate treatment have been prioritized more than cases which tend to have a progressive outcome and have no known therapies. Genetic research represents a beacon of hope for these patients, and their subsequent generations, so that gene therapy tailored to these patients can be developed. The technology for genetic analysis is already available; its use in ophthalmology is as yet negligible. Awareness and resources should be dedicated so that these analyses can be carried out, since there is so much that can be gleaned about the nature of these diseases from such research. We are looking to raise the awareness of the ophthalmological community in Bangladesh as a whole, so that there can be a hope for these patients in the near future.

Compliance with Ethical Requirements Authors Nahar N, Malek MIA, and Sarker BKD declare that they have no conflict of interest or any financial relationship to disclose. The study was cleared by the institutional ethical review committee at the Ispahani Islamia Eye Institute and Hospital (although being retrospective in nature, ethical approval was not mandatory).

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8 Genetic Research on Ocular Health and Disease in a Population from Nepal

Matthew P. Johnson, Suman S. Thapa, Sandra Laston, Kent L. Anderson, Bradford Towne, Janardan Subedi, John Blangero, and Sarah Williams-Blangero

Abstract

Ocular disease is a major public health concern worldwide, including Asia where large numbers of individuals suffer from blindness and visual impairment. In South Asian countries like Nepal, for example, age-related cataract is a leading cause of blindness and visual impairment. Age-related cataract is influenced by a complex interplay of non-genetic and genetic risk factors. Identifying the genetic risk factors involved can help elucidate the causal biological mechanisms underlying the development and progression of cataract and help identify those individuals at high risk of disease. The primary objective of an ongoing study in the Jirel ethnic group of eastern Nepal (the Jiri Eye Study) is to define the genetic architecture of normal ocular trait variation and characterize genetic factors influencing risk for common ocular diseases such as cataract. The Jirel population has been the focus of genetic epidemiological studies for more than two decades, and a well-documented extended pedigree has been developed for the group making it extremely powerful and informative for genetic studies. All 1292 study participants discussed in the work presented here belong to this single extended pedigree. Members of the Jirel population underwent a comprehensive eye examination that included lens opacity grading in accordance with the Lens Opacities Classification System II (LOCS II). A variance components method was used to estimate heritability of cataract. Of the 1292 participants, 57.0% were female and 43.0% were male. The mean (SD, range) age at exam is 42.0 (16.7, 18–88) years. The prevalence of cataract (any type) in individuals aged 40 years or older is 25.8%, and additive genetic effects (48.3%) play a significant role in determining the risk of developing cataract in this population. The Jirel population is a powerful resource for the study and identification of genetic mechanisms

M. P. Johnson (✉) · S. Laston · J. Blangero
S. Williams-Blangero
South Texas Diabetes and Obesity Institute,
Brownsville, TX, USA

Department of Human Genetics, School of Medicine,
University of Texas Rio Grande Valley,
Brownsville, TX, USA
e-mail: matthew.johnson@utrgv.edu

S. S. Thapa
Tilganga Institute of Ophthalmology,
Kathmandu, Nepal

K. L. Anderson
Department of Ophthalmology, School of Medicine,
UT Health San Antonio, San Antonio, TX, USA

B. Towne
Department of Population and Public Health
Sciences, Boonshoft School of Medicine,
Wright State University, Kettering, OH, USA

J. Subedi
Department of Sociology and Gerontology,
College of Arts and Science, Miami University,
Oxford, OH, USA

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influencing variation observed in ocular health and disease metrics. We anticipate that the Jiri Eye Study will continue to make significant contributions to the genetics of ocular health and disease in Asia.

Keywords
Cataract · Heritability · Pedigree · Nepal · Epidemiology

8.1 Introduction

Blindness and visual impairment (VI) are a global health concern that has a significant negative impact on the human condition. Worldwide, cataract is the leading cause of blindness and the second leading cause of VI (prevalence rates of 33.4% and 18.4%, respectively) [10]. The causative factors underlying blindness and VI differ across global regions, especially between developing and developed regions. South Asia is a developing region that includes Afghanistan, Pakistan, India, Bangladesh, Bhutan, and Nepal. Of 20 global regions examined, South Asia ranks fourth highest for age-standardized prevalence of blindness (4.4%) and highest for prevalence of VI (23.6%) [34]. Cataract is the leading cause of blindness and the second leading cause of VI throughout these countries in South Asia [10]. In Nepal, the prevalence of VI across several administrative zones has been reported to range from 18.6 to 43.0%, of which 0.7 to 17.4% is at least unilateral blindness [26–29, 36]. Consistent with what is observed throughout South Asia and across the world, cataract is a leading causes of blindness and VI in Nepal.

The high prevalence of blindness and VI due to easily correctable diseases like cataract in developing regions may, in part, be a consequence of the limited medical infrastructure and low numbers of trained medical personnel available to administer adequate ophthalmic care. In countries like Nepal, the difficulty in delivering adequate ophthalmic care is exacerbated by the remoteness of the areas in which most rural inhabitants live. Approximately 52% of the Nepalese population resides in the hill or mountainous regions of the country that are difficult or impossible to reach by motorized transport. Access to some areas is hampered seasonally during periods such as the monsoon when frequent landslides block major motorized thoroughfares and impede access to or delivery of ophthalmic care to those in need.

8.2 Age-Related Cataract: Clinical Features and Risk Factors

Age-related cataract (hereon, cataract) is an opacification of the natural crystalline lens, which impedes vision by perturbing the transmission and focus of light onto the retina. In a healthy lens, the cellular and protein material is arranged in a structured manner that allows light to pass through the transparent lens unobstructed. However, by the fifth or sixth decade of life, the cellular arrangement of the lens may start to lose its normal structure and proteins may start to aggregate into larger masses, thereby disrupting lens transparency [37].

There are three main types of cataract, which are defined by their clinical appearance and location within the lens [24, 37]. Nuclear cataract occurs when mature lens fibers at the center of the lens undergo oxidative stress which alters protein solubility and stability so that protein unfolding and aggregation occur, resulting in brunescence and opalescence. Additional peripheral layers of lens fibers surround central lens fibers leading to increased stiffness of the central fibers, i.e., hardening or sclerosis. Cortical cataract is defined by perturbations to the structure of mature fiber cells within the lens cortex (enveloping the lens nucleus) that may result in a loss of essential metabolites which in turn may promote protein oxidation and precipitation. Over time, discrete opacities may then develop in the cortex and extend in a “spoke-like” fashion toward the center of the lens. Posterior subcapsular cataract is characterized by granular, plaque-like opacities that form and accumulate in the central posterior cortex underneath the posterior capsule.

Cataract is a complex multifactorial disease with non-genetic and genetic risk factors. Advancing age is a risk factor, and females are at greater risk of developing cataract than males [24]. Exposure to ultraviolet B radiation (sunlight) and cigarette smoke (a preventable, modifiable risk factor) has been demonstrated to augment cataractogenesis, as has systemic diseases such as type 2 diabetes mellitus and hypertension [24]. Furthermore, cataract prevalence rates differ among ethnic groups (individuals of Asian ancestry tend to exhibit higher rates of cataract), which highlights a genetic component to cataract development and progression.

8.3 Age-Related Cataract: Genetics

Depending on the genetic model tested (additive, dominant) and cataract assessment (clinical or digital grades), genetic mechanisms influencing cataract risk have been estimated to contribute between 20% and 53% of the total phenotypic variation [15–18]. Despite this solid evidence for genetic risk factors influencing age-related cataract susceptibility, few genetic susceptibility loci have been identified (in contrast to the case for earlier onset, Mendelian forms of the disease) [31]. Examples of cataract susceptibility loci that have reached genome-wide significance include linkage regions mapped to chromosomes 1p14-p35 and 6p12-q12 in a cohort of Caucasian sibling pairs [20] and regions of linkage disequilibrium (association) mapped to chromosomes 3 (*KCNAB1* SNP rs7615568) and 21 (SNP rs11911275 upstream of *CRYAA*) in a multiethnic Asian (Malays, Indians, Chinese) cohort [23].

8.4 Mapping Genomic Regions Influencing Ocular Disease Susceptibility: The Power of the Pedigree

Family-based study designs that include relative pairs (e.g., siblings), nuclear families (e.g., trios or other parent-offspring configurations), and/or extended pedigrees provide optimal opportunities to decompose the genetic architecture of common complex phenotypes such as cataract or other multifactorial ocular diseases. These pedigree structures optimize the examination of genetic information such as heritability, linkage, and association within the cohort and are less prone to stratification phenomena that mask true genetic signals or generate false-positive signals (a potential limitation with study designs utilizing samples of unrelated individuals).

Of these three pedigree frameworks, the extended pedigree design provides the most power to differentiate between genetic and non-genetic effects, observe a greater number of meiotic events to localize genomic regions influencing the tested phenotype (linkage), and assess the association of sequence-specific variants within linkage regions that harbor causal genes. This is because the key advantage of an extended pedigree framework is the large number of relative pairs, of both close and distant kin, available for analysis. The power to detect linkage is maximized when individuals are concentrated into as few families as possible, and the greater the average pedigree size, the fewer the number of individuals needed to achieve the same power to detect linkage [2, 6].

With an emerging appreciation of the role of functional rare variants in common complex phenotypes [5, 14, 35], an extended pedigree study design also offers an optimal enrichment strategy to identify rare variants [38]. Mendelian mechanics suggests that an extended pedigree framework maximizes the chance of identifying rare variants sufficiently propagated from the pedigree founders down through the family lineage(s). This phenomenon allows for robust direct statistical testing while minimizing the influence of spurious linkage disequilibrium [7, 21]. Pedigree-based sequencing approaches also offer a more cost-effective strategy to catalog rare variants compared to a population-based (unrelated samples) strategy. Sequencing key family members (e.g., founders) allows for accurate pedigree-based imputation of non-sequenced family members utilizing phased (Mendelian-transmitted) sequence information to infer near exact

descendent haplotypes. This provides a more accurate determination of "whole" allele dosages (i.e., 0, 1, or 2 copies of the rare allele) compared to genome-wide association-based imputation utilizing population data.

The collection of extended pedigrees can be challenging. However, in areas of stable populations with limited migration rates, such as endogamous groups within South Asia [25], the ascertainment of large extended pedigrees is less of a challenge. Extended pedigrees from geographically and culturally isolated areas offer several significant advantages for disease gene identification compared to traditional, less homogeneous populations. These include a limited number of ancestors which minimize genetic and allelic heterogeneity and result in fewer susceptibility genes (with greater overall effect) and greater statistical power to identify these genes and reduced environmental "noise" which minimizes the confounding effects of non-genetic variables. These characteristics are of significant benefit for complex disease gene identification [9, 22, 32] and have proven effective in the identification of ophthalmic disease genes [3, 30].

Following the field-based data collection techniques for collecting large extended pedigrees in stable isolate populations outlined in Williams-Blangero and Blangero [47], we have successfully established several cohorts of extended pedigrees from populations in Alaska [19], Brazil [48], and Nepal (the Jirels) [49].

8.5 Genetic Epidemiological Studies in the Jirels of Nepal

8.5.1 Study Area

Nepal is divided into 14 administrative zones that are further subdivided into 75 districts. The Jiri region is located within the Dolakha District of Janakpur Zone and is approximately 190 km east of Kathmandu, the capital city of Nepal. Geographically, it is located at latitude 27°38'N and longitude 86°14′E, at an average elevation of approximately 2300 m above sea level, covering an area of approximately 230 km^2 bounded on the east and west by the Tamba Kosi and the Likhu Khola rivers, respectively. The region is named for the Jirels, a Tibeto-Burman language-speaking group.

8.5.2 Study Population

Approximately 2500 members of the Jirel population have participated in prior studies spanning a period of approximately 30 years. These studies include anthropological investigations [4, 42, 43], assessment of population structure [44–46], interrogating the genetic susceptibility to parasitic worm infection [49–52], and identifying genetic mechanisms influencing growth and development [40, 41]. The long-running research conducted in the region has resulted in the collection of extensive genealogical information on the Jirel people. All individuals who have previously participated in research studies belong to a single extended pedigree which makes this cohort an informative and extremely powerful resource for genetic studies [6, 47]. There is very little gene flow (<1% per generation) into the population from other ethnic groups, and inbreeding is minimized due to cultural rules [44]. Based on the strength and utility of this existing resource, we initiated a large-scale genetic epidemiological study of ocular traits and disorders in the Jirel population: the Jiri Eye Study (JES). The objective of JES is to characterize the genetic architecture of normal ocular trait variation and genetic factors influencing risk for ocular disorders such as cataract that are of major public health importance.

8.6 The Jiri Eye Study: Methods

8.6.1 Study Inclusion Criteria

The criteria for individuals to participate in JES are that they be in good health and are at least 18 years of age. Sex is not a selection factor, and we anticipate slightly more female than male volunteers based on prior recruiting experience.

Female volunteers who are pregnant defer their participation until at least 12 weeks postpartum.

8.6.2 Subject Recruitment

The target recruitment goal is 2000 individuals. Subject recruitment and examination activities take place during biannual visits to the field research site in Jiri (optimal data collection times are March/April and November/December). Prior to each field session, local recruitment staff contact potential study participants in person to explain the purpose and benefits of the study, provide them a copy of the Nepali-translated consent form (the consent form is read to individuals who are illiterate), obtain an individual's verbal agreement to participate in the study, and arrange an appointment date and time to attend the field research clinic that coincides with a forthcoming field site visit. Upon arrival to the clinic on their designated day, individuals are given time to reread the consent form or have it reread to them and to ask any questions they may have. Informed consent is documented by signature or thumb print (for illiterate individuals).

8.6.3 Questionnaire

A detailed interviewer-administered questionnaire is conducted in Nepali to collect self-reported information about systemic health (e.g., hypertension, diabetes), ocular health (e.g., previous trauma), and lifestyle factors such as cigarette smoking history.

8.6.4 Ophthalmic Examination

A comprehensive ophthalmic exam is conducted for all participants in the JES. It includes an external examination of the face and eyes, visual acuity assessment, slit-lamp biomicroscopy, Goldmann applanation tonometry, gonioscopy, fundus examination, fundus and optic disc photography, ocular biometry, Humphrey visual field assessment (SITA 24-2), pachymetry, and optical coherence tomography (anterior, posterior). Pertinent to cataract, lens opacity grading is conducted post-mydriasis (a mydriatic mixture of 1% tropicamide + 5% phenylephrine is administered) by comparing lenticular opacities with a standard set of images outlined in the Lens Opacities Classification System II (LOCS II) [13]. For the purposes of this analysis, we are interested in the general underlying genetic architecture (i.e., heritability) influencing cataract (any subtype) in the Jirel population. Therefore, the clinical phenotype is dichotomized into the presence of cataract (nuclear, cortical, and/or posterior subcapsular) in one or both eyes and absence of cataract in both eyes.

8.6.5 Statistical Methods: Heritability Estimate

The heritability estimate of cataract in the Jirel pedigree was determined using a variance components approach as implemented in SOLAR [1]. Our approach to estimate the narrow sense heritability (additive genetic effects), defined as $h^2 = \sigma^2_a/(\sigma^2_a + \sigma^2_e)$, fosters maximum likelihood methods that partition the observed phenotypic covariance (σ^2_p) into its additive genetic and environmental attributes. In its most simplistic, univariate form, the observed covariance of a quantitative trait in a pedigree of arbitrary size (n) is modeled as $\Omega = 2\Phi \times \sigma^2_a + I_n \times \sigma^2_e$, where Ω is the $n \times n$ covariance matrix, 2Φ is the $n \times n$ coefficient of relationship structuring matrix, σ^2_a is the variance in the observed trait due to additive genetic effects, I_n is the $n \times n$ identity structuring matrix for an implied individual-specific environmental component, and σ^2_e is the variance in the observed trait due to random (unmeasured) individual-specific environmental effects. Specific to this study, this model is easily extended to estimate the heritability of a dichotomous trait where we employ the liability and threshold model according to which it is assumed that dichotomous observations are underlain by a latent normally distributed liability and that individuals with liability values equal to or above a threshold on the liability scale express the

"affected" status and those falling below the threshold express the "unaffected" status [39]. The covariates age, sex and their interactions, and smoking (never, former, current) are also included in the additive genetic model. No bias of ascertainment correction is required since the Jirel family-based sample was originally obtained through house-to-house sampling, and the JES recruitment selection is not contingent on a specific ocular condition.

8.7 The Jiri Eye Study: Results

8.7.1 Power of the Jirel Pedigree

The theoretical framework underlying the analytical power calculation performed was developed by Blangero et al. [8] and took into account the exact structure of the Jirel pedigree. There is 80% power to detect an additive genetic effect (heritability) accounting for as little as 6.5% of the total phenotypic variation observed in the trait, in this case cataract.

8.7.2 Current Recruitment

To date, 1292 individuals (57.0% female) have successfully participated in the study. The age (in years) distribution is as follows: mean = 42.0, median = 40, standard deviation = 16.7, minimum = 18, and maximum = 88. With respect to cigarette smoking, 310 (24.0%) individuals never smoked, 749 (58.0%) individuals are former smokers, and 233 (18.0%) individuals are current smokers. Age-related ocular conditions generally start to manifest from 40 years of age. Therefore, pertinent to this age demographic in the Jirels, there are currently 666 individuals (53.5% female), and the age (in years) distribution is as follows: mean = 55.6, median = 54, and standard deviation = 10.9. The smoking summary relevant to this age group includes 211 (31.7%) individuals who never smoked, 250 (37.5%) individuals who are former smokers, and 205 (30.8%) individuals who still currently smoke.

8.7.3 Current Jirel Pedigree Structure

The current Jirel pedigree framework is comprised of 17,343 pairings of relatives of varying degrees of relationship to each other. These relative pair relationships include 709 parent-offspring pairs, 669 sibling pairs, 117 grandparent-grandchild pairs, 1319 avuncular pairs, 106 half-sibling pairs, 26 double first-cousin pairs, 2508 third-degree relative pairs, 3480 fourth-degree relative pairs, 3801 fifth-degree relative pairs, 2758 sixth-degree relative pairs, 1633 seventh-degree relative pairs, 196 eighth-degree relative pairs, and 21 ninth-degree relative pairs.

8.7.4 Cataract Distribution

The distribution of cataract (any of the three subtypes) in one or both eyes is presented in Table 8.1. No individual younger than 40 years of age was diagnosed with cataract; therefore, the percentages presented in the table are calculated for the subset of individuals in the 40+ years' age group ($n = 666$).

8.7.5 Cataract Heritability Estimate

The heritability of cataract modeled as a dichotomous trait in the Jirel pedigree was significant ($h^2 = 0.493$; $p = 0.046$; SE = 0.292). For the liability and threshold model (dichotomous observations underlain by a latent normally distributed

Table 8.1 Cataract distribution in the Jirel pedigree

		n	%
Age	40–49	5	0.8
	50–59	22	3.3
	60–69	75	11.3
	70–79	61	9.2
	80–89	9	1.4
	Total	*172*	*25.8*
Sex	Male	72	10.8
	Female	100	15.0
	Total	*172*	*25.8*

liability), the nearest equivalent to a measure of the explained phenotypic variance due to covariates is the Kullback-Leibler divergence (KLD) [11]. The KLD measures the proportionate reduction in uncertainty to the covariates in the model which, for cataract, is 0.623. Estimating heritability of cataract in the 40+ years' age group alone (n = 666) did not significantly alter the result (h^2 = 0.483; p = 0.048; SE = 0.292; KLD = 0.513). This result highlights evidence of a genetic contribution (approx. 50%) to influence the risk of age-related cataract in the Jirel population. Therefore, further investigation is warranted to localize genomic regions (linkage) and identify common and/or rare sequence-specific variants within these linkage regions (association) that harbor causal genes influencing age-related cataract development and progression.

8.8 Comment

We report, for the first time, data on cataract from the Jiri Eye Study, an extended pedigree-based genetic epidemiological investigation of the determinants of ocular traits and disorders in the Jirel ethnic group of eastern Nepal. The Jiri Eye Study aims to document the distribution and prevalence of standard ocular biometry and ocular disease in addition to characterizing the genetic architecture of normal ocular trait variation and genetic risk factors for ocular diseases of global public health importance.

We found that the crude prevalence of any type of age-related cataract (25.8%) for individuals aged 40 years or older in the Jirels is in general agreement with rates seen in other Asian countries (8.3–61.9%) [12, 53], including Nepal (10.2–31.5%) [33]. The preponderance of Jirel females (15.0%) with cataract, compared to Jirel males (10.8%), also is in agreement with other reports documenting female sex to be a risk factor [24].

Our results suggest a significant genetic contribution (48.3%) to risk for cataract in the Jirels and an almost equal contribution (51.3%) of non-genetic factors (age, sex, their interactions, and cigarette smoking) influencing cataract risk in this population. While the genetic contribution to cataract risk in the Jirels is similar to other reported heritability estimates, we must note that our estimate is for any type of cataract, whereas the studies by Hammond et al. [17, 18] and Congdon et al. [15, 16] report heritabilities between 20% and 53% for specific cataract subtypes. Variation in heritability estimates for age-related cataract may result from different study designs (extended pedigree, twins, sibling pairs, respectively), ethnic differences (South Asian, Caucasians, Caucasian and African American, respectively), and possibly the mean age of individuals presenting with cataract (~55 years, ~62 years, ~75 years, respectively). The subjective nature of our LOCS II cataract grading scheme will also likely produce nuances not only in our cataract heritability estimates but also Jirel cataract prevalence rates when compared to other studies.

In conclusion, we have demonstrated for the first time that the prevalence of age-related cataract in the Jirel population of Nepal is high (25.8%) and that additive genetic effects (48.3%) play a significant role in risk of developing this condition, which has a significant negative impact in Nepal, other Asian countries, and around the world. The Jirel population is ideally suited for genetic epidemiological studies due to its genetic isolation and deep genealogical relationships which augment the power to localize genomic regions and identify sequence-specific variants in causal genes influencing gene networks related to ocular biology. With data from a comprehensive eye examination (anterior, posterior) being conducted in the Jirel population, coupled with an existing set of genome-wide SNP data and a prospective catalog of whole-exome sequence variants, the Jiri Eye Study will continue to make significant contributions to the understanding of the genetics of ocular health and disease in Asia.

8.9 Summary

To our knowledge this is the first large-scale genetic epidemiological study of ocular traits and disorders conducted in Nepal, and it is also the

first study to estimate the prevalence of blindness, visual impairment, and their causes in the Jirel population. We suspect that this may well be the first eye examination for many of the study participants. This study will impart knowledge about ocular health and disease in the Jirel ethnic group which, in turn, will help the implementing body of the Nepal Eye (health) Program, the Tilganga Institute of Ophthalmology, plan for the future development of eye care infrastructure and public health programs. This ocular health knowledge will not only help the Jirel people and the Municipality of Jiri but also the Nepalese population in general. For the study participants themselves, an immediate benefit includes provision of sight-correcting glasses, an effective and inexpensive strategy to improve vision and reduce its burden on the individual and society. Overall, data from this project will contribute to our understanding of the determinants of blindness and visual impairment in a novel Asian population, a necessary step if we are to significantly reduce its negative impact on human well-being.

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Compliance with Ethical Requirements All procedures were in accordance with the ethical standards of and approved by the University of Texas Rio Grande Valley Institutional Review Board (IRB# 2016-093-05) and the National Health Research Council of Nepal (Reg. No. 177/2014). Procedures were in accordance with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all individuals prior to participating in the study. All authors declare that they have no conflict of interest.

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9 Genetic Eye Research in the Philippines

Patrick R. Ching, Edward Ryan A. Collantes, Michelle D. Lingao, Patricia E. Cabrera, and Leo D. P. Cubillan

Abstract

The Philippines is a developing nation in Southeast Asia and is home to over 100 different ethnolinguistic groups. As in other countries, genetic eye diseases in the Philippines are rare but collectively represent a significant cause of blindness. Although specialized facilities providing genetic services in the country exist, only a handful of genetic eye studies have been done in the Filipino population. Training more Filipino scientists and ophthalmologists in the field of genetic eye research can lead to more discoveries, collaborations, and increased representation of Filipinos in population-based genetic studies. Through evidence-based policies and programs, healthcare providers and public health practitioners with the involvement of government and industry can use genomic knowledge for health promotion and disease prevention.

Keywords

Philippines · Genetics · Eye diseases · Public health

P. R. Ching
Departments of Biostatistics and Epidemiology, Harvard TH Chan School of Public Health, Boston, MA, USA

Center for Evidence-Based Imaging, Brigham and Women's Hospital, Boston, MA, USA

E. R. A. Collantes (✉)
Massachusetts Eye and Ear, Harvard Medical School, Manila, Philippines

Manila Doctors Hospital, Manila, Philippines
e-mail: ryan_collantes@meei.harvard.edu

M. D. Lingao
Department of Ophthalmology and Visual Sciences, University of the Philippines Manila, Manila, Philippines

Asian Eye Institute, Makati, Philippines

P. E. Cabrera · L. D. P. Cubillan
National Institutes of Health, Philippine Eye Research Institute, University of the Philippines Manila, Manila, Philippines
e-mail: leo.cubillan@up.edu.ph

9.1 The Philippines

The Philippines is an archipelago in Southeast Asia. Located in the western Pacific Ocean, it is composed of 7641 islands [1] that belong to three island groups: Luzon, Visayas, and Mindanao. It is bounded by the Philippine Sea to the east, Celebes Sea to the south, Sulu Sea to the southwest, and South China Sea to the north and west. With Manila as the capital, the Philippines has a total land area of about 300,000 km^2.

According to the World Bank [2], the Philippines continues to be the 12th most populated country in the world with a population of 100.98 million and population density of 337 persons/km^2 in 2015 [3]. The average annual population growth rate is 1.7% during

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the period of 2010–2015 [3]. Of the total population, 50.6% was male and the median age was 24.3 years. The working age group belonging to ages 15–64 years made up 63.4% of the population; children below 15 years accounted for 31.8% and those aged 65 years and above comprised 4.7%. The average household size in 2015 was 4.4 persons [3].

Through the years, health indicators have improved. In 2015, the average life expectancy among Filipino men and women was 65 and 72 years, respectively (both sexes, 69 years) [4]. The crude birth rate (per 1000 population) was 24.4 and the crude death rate (per 1000 population) was 5.9 [5]. Adult mortality rate (the probability of dying between 15 and 60 years of age per 1000 population) for men was 255 (from 272 in 1990) and for women was 136 (from 154 in 1990) [5]. Maternal mortality ratio decreased from 152 to 114 per 100,000 live births from 1990 to 2015. A decline in infant mortality rate was also seen from 41.1 deaths per 1000 live births in 1990 to 23.5 deaths per 1000 live births in 2013 [5].

Total health expenditure (THE) increased from PHP530.3 billion in 2013 to PHP585.3 billion in 2014, a growth of 10.4% [6]. While 4.6% of the country's gross domestic product (GDP) went to THE, the Philippines spent 0.1% of its GDP for scientific research and development in 2014 and 0.14% in 2016 [7]. Although the country remains among the fastest growing Southeast Asian economies, 21.9 million Filipinos were still living in poverty in 2015 [8].

With over 100 distinct ethnolinguistic groups, the Philippines is a diverse country – geographically, linguistically, and culturally. Foreign influences come from various parts of Asia, Europe, and the Americas. According to a population-based genetic study of Filipinos, it was determined that Philippine groups shared genetic affinities with groups from South Asia and Australia [9]. There are however much closer genetic affinities with groups from Southeast Asia and Taiwan.

9.2 Burden of Eye Disease

According to the Third National Survey of Blindness in the Philippines conducted in 2001–2002 [10], the nationwide prevalence of visual impairment (visual acuity worse than 6/18 in the better eye) was 4.62%. Bilateral and unilateral low vision (visual acuity worse than 6/18 but equal to or better than counting fingers at 3 m) had prevalence of 1.43% and 0.87%, respectively, with error of refraction as the leading cause (53%). The prevalence of bilateral and unilateral blindness (visual acuity worse than counting fingers at 3 m) was 0.58% and 0.71%, respectively. Cataract (62.1%) was the most common cause of blindness, followed by error of refraction (10.3%), glaucoma (8%), retinopathy (4%), and maculopathy (4%). In a more recent study among Filipinos in the working age group, it was found that the most common causes of blindness or severe visual impairment were diabetic retinopathy, pathologic myopia, hereditary retinal disorders, glaucoma, and optic atrophy [11].

In 2016, cataract (28.2%) and errors of refraction (26.8%) were the two most common diagnoses at the ophthalmology outpatient department of the Philippine General Hospital (PGH), the largest tertiary state-owned training hospital in the country. Eye diseases with presumed genetic etiologies made up approximately 1% of the cases, with retinitis pigmentosa as the most common one. Other conditions seen include inherited and galactosemia cataract; optic nerve, iris, and chorioretinal colobomas; congenital fibrosis of extraocular muscles; congenital nystagmus; Peter's anomaly; and ocular albinism, among others [12].

In the Philippines, as in other countries, genetic eye diseases are rare but collectively represent a significant portion of blinding eye conditions. The prevalence of inherited eye diseases in the country is not known because they are grouped under broad categories in epidemiologic studies [10, 13]. Genetic diseases as a whole are underreported in the country, partly because of the limited number of trained geneticists who can provide specific diagnoses and partly because of the high

cost of establishing molecular diagnoses. With the current developments in treatment for genetic eye diseases, interest in obtaining molecular diagnoses for these conditions is increasing. Referrals to genetic specialists are also expected to increase.

9.3 Genetic Eye Disease Studies in Filipinos

9.3.1 Visual Screening in the Aeta Population

A Philippine ethnolinguistic group called the Aeta are hunter-gatherers who have a pygmy phenotype [9]. The Aeta reside in the northeastern part of Luzon island where they mainly practice swidden agriculture [13]. DNA analysis revealed that some Aeta groups are genetically affiliated with the Indian population, while another group of Aeta is closer to Southeast Asian and Oceanian groups.

There was an interest in studying the visual function of this population because they had never been examined in the past and as a consequence of their relative isolation had been outside the scope of standard medical care [14]. Visual screening in 225 individuals belonging to the Aeta population was implemented, and a standard ophthalmologic examination was performed including visual acuity, intraocular pressure, gonioscopic, and dilated fundus examination. The major causes of blindness were cataract and refractive error in 86% of patients. Other ocular conditions observed in this study population were pterygium, corneal scarring, penetrating trauma, exfoliation syndrome, and retinal disease. There were no definite cases of primary open-angle glaucoma and closed-angle glaucoma identified. The absence of glaucoma in this study population may be due to chance or, more interestingly, differences in genetic susceptibility or environmental factors that lower the risk for primary forms of glaucoma in these populations. Visual function screening in other ethnolinguistic groups in the Philippines may shed light on these unique populations and the various eye conditions they may or may not have.

9.3.2 Prevalence of Color Vision Deficiency in Filipino High School Students

Color vision deficiency is one of the most common disorders of vision and can be divided into congenital and acquired forms [15]. The most common color vision defects, commonly (and incorrectly) termed color blindness, are congenital [16]. A cross-sectional study was conducted among 1258 male Filipino high school students to screen for color vision deficiency. The prevalence of color vision deficiency was found to be 5.17% of the total number of students screened with the deutan type, the most common type in this study population [17].

9.3.3 Juvenile Open–Angle Glaucoma in Filipino Families

Glaucoma is the most common cause of irreversible blindness worldwide [18]. It is projected that by the year 2020, open-angle glaucoma will affect 58.6 million individuals [19]. Juvenile open-angle glaucoma (JOAG) is a primary open-angle glaucoma characterized by disease onset before the age of 35, high intraocular pressure, and a normal appearance of the drainage angle at gonioscopy. JOAG follows an autosomal dominant inheritance pattern [20]. Myocilin (MYOC) gene mutations lead to JOAG by causing the myocilin protein to become misfolded. The misfolded protein is then sequestered in the endoplasmic reticulum (ER) in trabecular meshwork (TM) cells eventually leading to ER stress-induced TM cell death [21–23].

Genetic studies have been conducted on Filipino families with JOAG. The MYOC gene was screened for mutations in a family with an autosomal dominant form of JOAG [24]. No MYOC mutations were found in this family. Linkage analysis was then performed which showed mapping of a novel genetic locus at 5q22.1-q32 [25], necessitating further studies on this genomic region. More recently, a novel MYOC nonstop mutation, c.1515A>G (p.*505Wext*42), was identified in another

Filipino family [26]. Functional studies suggest that this mutation causes sequestration of the MYOC protein in the ER similar to cells transfected with a known MYOC mutation c.734G>A (p.C245Y) [27].

9.3.4 Eye Movements in X–Linked Dystonia with Parkinsonism

X-linked dystonia-parkinsonism is an X-linked recessive syndrome of combined dystonia-parkinsonism [28]. The underlying genetic cause has not been fully elucidated although efforts to determine this are currently underway. All XDP cases described have so far been linked to Filipino ancestry with an especially high prevalence in the island of Panay. The prevalence of XDP in the Philippines is 0.31 per 100,000 and in Panay island 5.74 per 100,000 [29].

XDP manifests predominantly as torsion dystonia, later combined with or sometimes replaced with parkinsonism [29]. Eye movements in patients with XDP have also been described [30, 31]. XDP patients were found to have Parkinson-like oculomotor dysfunctions with normal main sequence, reduced saccade and smooth pursuit gain and normal horizontal saccade latency. Further studies on the prevalence of ocular movement abnormalities and its correlation to disease severity in XDP patients may aid in better characterization of the disease as well as potential markers of disease status and prognosis.

9.3.5 Molecular Profiling of Retinoblastoma

Retinoblastoma (RB) is the cause of approximately 4% of all pediatric malignancies worldwide [32]. As the most common intraocular malignancy in children, the estimated incidence of RB in the Philippines is 237 in 100,000 [33]. There is a 7–8% familial incidence among RB patients, similar to prevalence rates in other countries [34].

Molecular profiling of RB tumor samples was done in a cohort of Filipino patients [35]. A known nonsense mutation in exon 18 of the RB1 gene was detected in one tumor sample, while a novel missense mutation in exon 19 was identified in another tumor. Further studies are needed to confirm if this novel mutation is disease causing.

9.3.6 Case Reports of Rare Ocular Disorders

There have been numerous case reports on various genetic eye diseases as well as systemic syndromes with eye manifestations in Filipinos. Several families with retinitis pigmentosa were screened for mutations in rhodopsin and peripherin/RDS genes [36]. No mutations were identified in these patients, necessitating follow-up studies to screen for other genes which cause inherited retinal diseases. In patients with mucopolysaccharidosis type II, it was found that 40% of them had errors of refraction, while 20% had retinal pigmentary changes [37]. Patients clinically characterized as having Usher syndrome [38], Alport syndrome [39], Bardet-Biedl syndrome [40], Waardenburg syndrome type 1 [41], and encephalocraniocutaneous lipomatosis [42] have also been reported. With the establishment of molecular diagnostic facilities in the country, it is with great hope that future case reports will be accompanied by a genetic diagnosis to get a better understanding of the disease as well as the possibility of offering gene-based treatments to these patients.

9.4 Genetic Services in the Philippines

The Institute of Human Genetics-National Institutes of Health (IHG-NIH) at the University of the Philippines (UP) Manila is the largest provider of genetic services in the country. It provides clinical, diagnostic, and research services including clinical genetics, cytogenetics, molecular genetics, biochemical genetics, and newborn screening. It has a Clinical Genetics Unit (CGU) which provides comprehensive clinical evaluation, appropriate management, and genetic

counseling services to persons and families with or at risk for an inherited condition [43]. The CGU offers karyotyping, high-resolution banding, and fluorescence in-situ hybridization analyses for different chromosomal disorders and neoplasms.

The Molecular Genetics Unit is involved in research utilizing molecular-based tools and techniques for unique and common genetic diseases among Filipinos. Focused on the diagnosis and management of inherited metabolic disorders, the Biochemical Genetics Unit conducts biochemical tests including urine metabolic screen, urine organic acid analysis, quantitative amino acid analysis, etc. It also houses the first Newborn Screening Center (NSC) in the country that provides an advanced newborn screening for more than 20 disorders [43].

Aside from IHG-NIH, there are other NSCs in the country and institutions that offer cytogenetic testing and clinical genetics services [43]. The Department of Ophthalmology and Visual Sciences at the PGH and the ocular genetics department at the Asian Eye Institute, a privately owned ambulatory eye center in the Philippines, provide comprehensive ophthalmic care, diagnostic evaluation, testing, and counseling for patients with genetic eye diseases.

Additionally, the Philippine Genome Center (PGC) is a multidisciplinary institution that seeks to develop genomics research in the country. With health as one of its priorities, the PGC aims to apply genomics to both clinical and public health practice for population benefit. Some of the projects in the health program include web-based genome library for influenza virus (H1N1) and development of diagnostic kits for early diagnosis of infectious diseases and noncommunicable diseases [43].

9.5 Education and Training

Strengthening the academic and research infrastructure requires more collaboration among the academics, government, and private industries. As the PGC implements research program-driven agenda on priority areas, it establishes institutional linkages within and outside the country that facilitate the sharing of technology and expertise in genomics. In addition, active promotion of genomics competencies among health professionals needs to be emphasized as it remains essential in the translation of genomics to clinical and public health practice.

In the Philippines, there are only a few medical geneticists. While only PGH offers fellowship training in clinical genetics, only a handful of institutions have graduate programs for genetics, molecular medicine/biology, or biomedical informatics.

Although the number of Filipino scientists involved in medical genetics, genomics, bioinformatics, and related fields has increased through the years, the need for more researchers trained in these fields still exists. Experts in genetic epidemiology, quantitative genetics, and computational biology can mentor students and young investigators, and this can lead to more genetic knowledge and discoveries in ophthalmology and other areas of medicine. Geneticists, traditional and genetic epidemiologists, biostatisticians, bioinformatics specialists, and public health practitioners have much to learn from one another. In this era of big data where rapid technologic advances continue, Filipino researchers should also explore opportunities in other omics fields like epigenomics, transcriptomics, proteomics, and metabolomics.

9.6 Genomics for Health Promotion and Public Health

Advances in genomic eye research must be translated to public health. As the knowledge of the disease biology of different medical conditions increase, healthcare systems become better equipped to modify their prevention strategies. Sound health policies must be guided by scientific research and community consultation. Public health programs borne out of these must be continuously assessed. While advances in public health do not correspond to the developments in genomics over the recent years, creation of policies for genetic testing and other services should

nevertheless be emphasized. As Brand and colleagues said, a conceptual shift in both medicine and public health is needed with the increasing genome-based knowledge: genomics needs to understand how it can include public health aspects in its work program while public health needs to analyze how genomics changes the concepts of public health [44].

It is important to recognize that genomic variation is vital in understanding disease, their impact on health, and how we understand differences in ancestral populations. Genome-wide association studies (GWAS) have played a major role in our understanding of variants involved in disease as well as understanding ancestral differences. It is worth noting however that 81% of GWAS studies involve subjects who are of European ancestry [45]. These figures have improved throughout the years, with representation from those of Asian ancestry participating in GWAS steadily increasing from 3% in 2009 to 14% in 2016. In order to improve the diversity in genetic databases, various Asian populations like the Philippines should be more involved. The infrastructure to diagnose and study genetic eye diseases is already present in the country. The next logical step is to conduct more robust research as well as establish relationships with international collaborators especially in the Asia Pacific region.

9.7 Summary

To date, only a few genetic studies in eye diseases have been conducted in the Philippines. Although only a few causal genetic variants have been identified, genetic studies have been extremely useful in identifying new biological pathways that should be considered in the prevention or treatment of eye diseases. More support from the government and funding agencies will encourage more genetic studies that can yield deeper insights into disease biology and therapy.

Despite the significant increase in the proportion of genome-wide association study participants who are of Asian ancestry from 3% in 2009 to 14% in 2016 [45], better representation of groups of non-European descent is still necessary. Since genomic research infrastructure to diagnose and study genetic eye diseases is already present in the Philippines, the next logical step is conducting more robust investigations. Through international collaborations especially in the Asia Pacific region, efficient generation of research findings with better accuracy, relevance, and generalizability including the vulnerable and underserved will be within reach.

Results should be made available to healthcare providers and the public since these will guide management of eye conditions, and, through shared decision-making, the latter will be able to make better informed choices. Moreover, discoveries from these studies will help public health officials and lawmakers in planning, implementing, and evaluating public health policy. This underscores the need to strengthen the genetics, genetic epidemiology, and bioinformatics training in the country. As computerized and online systems, biobank, and other genomic infrastructure become more available, our researchers should take full advantage of these to inform prediction, screening, prevention, and intervention strategies. Knowledge from genetic and genomic studies should not only benefit developed nations; it should improve the health of all people.

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

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Hereditary Eye Disease in Ningxia Hui Autonomous Region of China

10

Weining Rong, Huiping Li, and Xunlun Sheng

Abstract

Ningxia Hui Autonomous Region is the main gathering region of Hui nationality in China. Because of unique geographical environment and national characteristics, the incidence of hereditary eye disease is higher in Ningxia Region. From 2010 to 2017, our research team collected 230 pedigrees and 268 sporadic patients with monogenic hereditary eye disease, including 210 pedigrees and 250 sporadic patients with hereditary retinal diseases. Among these pedigrees, 89 (42%) families reported a family history of consanguinity. We carried genetic research on 113 pedigrees and 154 sporadic patients with hereditary retinal diseases. The disease-causing mutations were identified in 66 pedigrees and 69 sporadic patients. Total 63 related disease-causing genes were involved. Four new disease-causing genes were discovered through whole exome sequencing combined with a series of cell experiments and animal experiments, including GUCA1A gene that causes central areolar choroidal dystrophy, CCT2 gene that causes Leber congenital amaurosis, CEP78 gene that causes Usher syndrome, and PRPF4 gene that causes autosomal dominant retinitis pigmentosa. Through whole exome sequencing, we confirmed the clinical diagnosis of combined Marfan syndrome with X-linked hypophosphatemia in a pedigree with complex phenotypes. The homozygous mutation of BEST1 gene was found in a consanguineous family with complicated phenotype, and the diagnosis of autosomal recessive bestrophinopathy (ARB) was made.

Keywords

Ningxia · Ocular disease · Genetic · Gene · Mutation · Consanguineous marriage

10.1 Introduction

Ningxia Hui Autonomous Region is located in the northwest of China. Ningxia covers a total area of 66,400 square kilometers. The total population is 6.82 million in 2017, of which the Hui people accounts for 35.6%. The Hui nationality religion is Islam. Ningxia is one of the five minority autonomous regions in China. It governs 5 prefecture-level cities and 22 counties (or county-level cities, districts).

Compared with other provinces of China, the economy of Ningxia is relatively backward. Many counties are located in remote mountainous areas with low traffic and population mobility, little communication with the outside world, and low cultural quality of the population. Early

W. Rong · H. Li · X. Sheng (✉)
Ningxia Eye Hospital, Ningxia People's Hospital, Ningxia, China

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marriage and consanguineous marriage are extremely serious. In 2000, a survey about consanguineous marriage of rural Hui population in Ningxia showed that the consanguineous marriage rate was 46%. Therefore, the incidence of hereditary diseases in Ningxia is very high.

Our study conformed to the Declaration of Helsinki and was approved and prospectively reviewed by the Ethics Committee of Ningxia People's Hospital.

Since 2002, we have carried out research on hereditary eye disease in Ningxia. In our study, all procedures followed were in accordance with the ethical standards of the Ethics Committee of Ningxia People's Hospital on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study. After more than 10 years of research, we have made some achievements in the field of hereditary retinal diseases. From 2010 to 2017, our research team collected 230 pedigrees and 268 sporadic patients with monogenic hereditary eye disease, including 210 pedigrees and 250 sporadic patients with hereditary retinal diseases. Among these pedigrees, 89 (42%) families reported a family history of consanguinity. The consanguinity marriages in 72 families were between first cousins, and 9 families were between second cousins; only 5 families were between half cousins matrimony (2). The families we collected mainly include the following diseases: retinitis pigmentosa (RP), cone degeneration (COD), cone-rod degeneration (CORD), Leber congenital amaurosis (LCA), Stargardt disease (STGD), bestrophinopathies including best vitelliform macular dystrophy (BVMD) and autosomal recessive bestrophinopathy (ARB), occult macular dystrophy (OMD), achromatopsia, congenital stationary night blindness (CSNB), choroideremia (CHM), gyrate atrophy of the choroid and retina, Usher syndrome, Bardet-Biedl syndrome, Crouzon syndrome, ocular albinism, Oliver-McFarlane syndrome, central areolar choroidal dystrophy (CACD), microcornea, Leber hereditary optic neuropathy, Sveinsson's chorioretinal atrophy, blepharophimosis-ptosis-epicanthus inversus syndrome (BPES), congenital cataract, Marfan syndrome (MFS), Weill-Marchesani syndrome, Axenfeld-Rieger syndrome, dominant optic atrophy, recessive optic atrophy, and X-linked juvenile retinoschisis (XLRS).

We carried genetic research on 113 pedigrees and 154 sporadic patients with hereditary eye diseases. The disease-causing mutations were identified in 66 pedigrees and 69 sporadic patients; the positive rate was 58% and 45%, respectively. Total 63 related disease-causing genes were involved. About 60% of patients with retinitis pigmentosa are recessive; the mutation rate of USH2A gene was the highest, followed by MYO7A gene in RP pedigrees and C2orf71 gene in sporadic RP patients. In other hereditary retinal diseases, ABCA4 gene mutation rate was the highest, followed by CRB1 gene. Seven mutations were detected in 7 families with Marfan syndrome, and 15 mutations on mitochondrial gene were detected in 15 Leber hereditary optic neuropathy (LHON) patients.

Four novel disease-causing genes were discovered through whole exome sequencing combined with a series of cell experiments and animal experiments, including GUCA1A gene that causes central areolar choroidal dystrophy, CCT2 gene that causes Leber congenital amaurosis, CEP78 gene that causes Usher syndrome, and PRPF4 gene that causes autosomal dominant retinitis pigmentosa. Through whole exome sequencing, we confirmed the clinical diagnosis of combined Marfan syndrome with X-linked hypophosphatemia in a pedigree with complex phenotypes. The homozygous mutation of BEST1 gene was found in a consanguineous family with complicated phenotype, and the diagnosis of autosomal recessive bestrophinopathy (ARB) was made. We will describe the typical cases one by one.

10.2 Central Areolar Choroidal Dystrophy (CACD)

10.2.1 Introduction

Central areolar choroidal dystrophy (CACD), first described in consanguineous marriage fam-

ily [1], is a clinically and genetically heterogeneous pigmentary retinopathy characterized by fine, mottled depigmentation in the macular area. Later, atrophy of the retinal pigment epithelium and choriocapillaris is observed [2]. It usually shows progressive and profound visual loss between 30 years and 60 years of age [3, 4]. It may be challenging to diagnose CACD in the early stages of the disorder because of the nonspecific depigmentation. Also, the late-onset CACD due to PRPH2 mutation which occurs by the seventh decade share common features with age-related macular degeneration (AMD), including choroidal neovascular and drusen lesion, and may easily be misdiagnosed [5–7]. The relationship between genotype and phonotype of CACD is not fully elucidated. CACD consist of 8% hereditary retinal dystrophies [8]. Epidemiological study base on population is limited due to rare occurrence.

10.2.2 Pathology

The pathology of CACD is characterized by absence of photoreceptors, RPE, and choriocapillaris in the area of atrophy, which coincides with clinical findings.[2] Thinning to complete loss of the outer nuclear layer in optical coherence tomography (OCT) examination can be observed in the CACD process [4]. Previous studies showed that lipofuscin accumulated in the subretinal space where RPE cells were distorted and expanded that resulted in choriocapillaris atrophy, but overlying photoreceptors were partly atrophic. Recently, our study renewed this concept. We found a novel mutation GUCA1A p.R120L in a five-generation family with CACD. The overexpression of p.R120L mutation of GUCA1A in zebrafish resulted in a significant degeneration of both rod and cone cell; also the RPE is impaired (Fig. 10.1) [9]. We propose a hypothesis that degeneration of photoreceptor was primary insult for CACD; overaccumulation of OS debris or other abnormal substances derived from those dying photoreceptors are toxic to the RPE, which can lead to RPE impairment; and RPE will in turn aggravate photoreceptor degeneration. The choriocapillaris atrophy following RPE degeneration is a result of a lack of growth factors secreted by the RPE, which are essential for choroidal vasculature homeostasis. This may be more reasonable to explain the lipofuscin accumulated beneath the retina.

10.2.3 Genetic Aspects

All genes associated with CACD are photoreceptor-specific. Autosomal dominant CACD is most commonly caused by mutations in the peripherin-2 (PRPH2) gene and GUCY2D gene [3, 9–12]. To date, eight different mutations in the PRPH2 gene [5, 11, 13–17] have been identified to cause the CACD phenotype. The most frequently detected mutation in PRPH2 is R172W, which has been reported in different ethnic groups. Recently, our investigation reveals that a novel GUCA1A mutation p.R120L contributes to CACD etiology in dominant trait in a five-generation family (Fig. 10.1) [9]. Clinically CACD mainly affects RPE and choriocapillaris, but GUCA1A mutation results in photoreceptor dystrophy initially, followed by RPE and choriocapillaris atrophy. The diverse pathogenic mechanisms of GUCA1A mutation may imply that the photoreceptor degeneration which might be neglected previously triggers the cascade of RPE and choriocapillaris defect in another two genes of CACD. Similar to our cases, overexpressing mutated PRPH2 p.R172W also impaired photoreceptors of mice retina and secondary RPE/choriocapillaris degeneration [18].

10.2.4 Clinical

Four clinical stages of CACD have been described [2, 3]. In stage I CACD, slight and focal parafoveally pigmentary RPE changes can be observed, usually in the adolescent patient. Stage II is characterized by oval-to-round, mildly atrophic, hypopigmented area, which shows speckled FAF pattern on a fundus autofluorescence (FAF) image, usually 1.5 to several disc diameters. In stage III one or more patches of

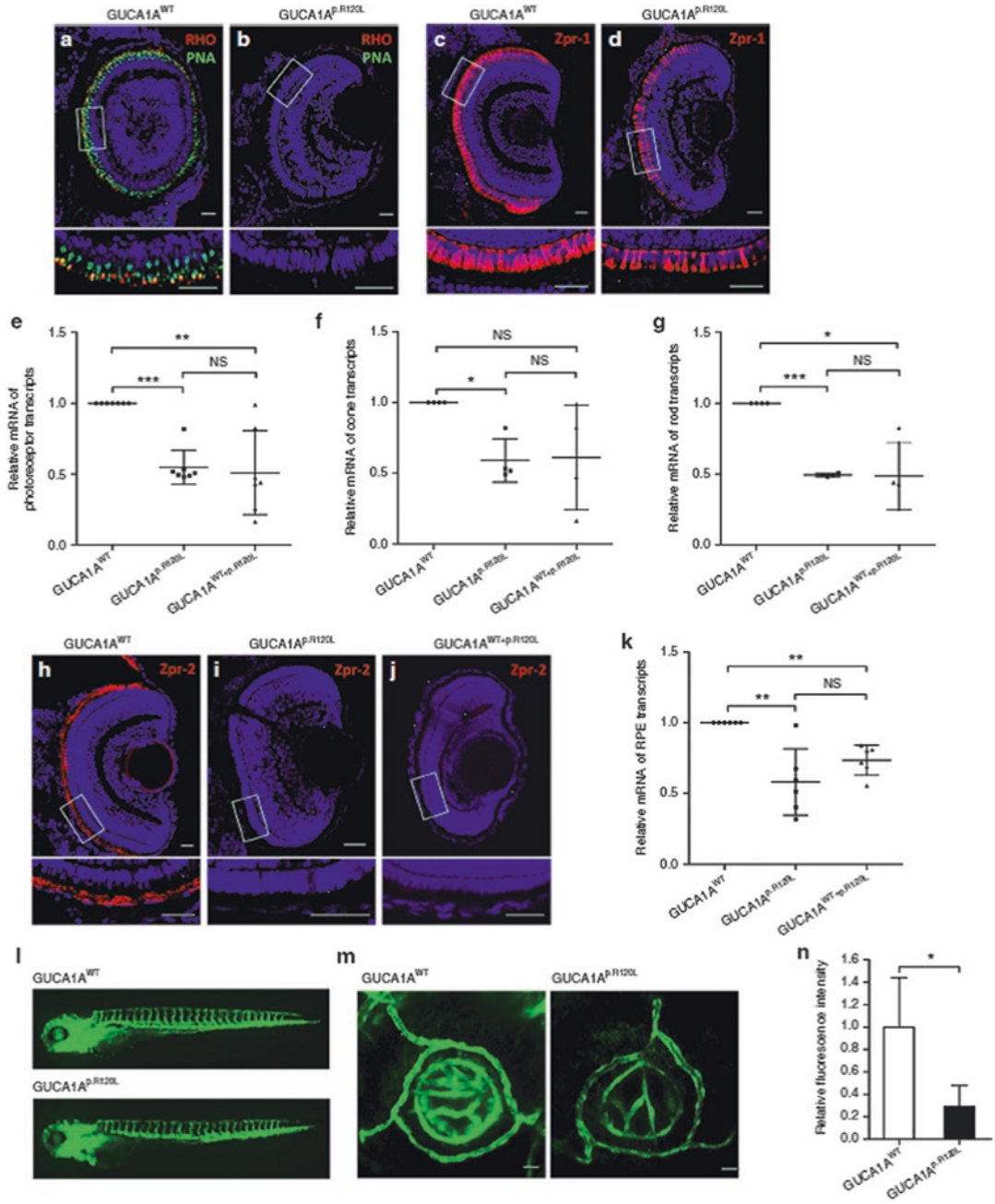

Fig. 10.1 Impairments in photoreceptors, the retinal pigment epithelium (RPE), and ocular vasculature induced by GUCA1A p.R120L in zebrafish. (**a**–**d**) Immunofluorescent staining of rhodopsin (RHO) (red; **a**, **b**), peanut agglutinin (PNA) lectin (green; **a**, **b**), and Zpr-1 (red; **c**, **d**). RHO and PNA expressions were significantly reduced in zebrafish overexpressing GUCA1Ap.R120L (**a**, **b**). Zpr-1 expression was only slightly reduced in the GUCA1Ap.R120L-injected group (**d**) when compared with GUCA1AWT-injected zebrafish (**c**). (**e**–**g**) Relative messenger RNA (mRNA) levels of photoreceptor-, cone-, and rod-specific transcripts in GUCA1Ap.R120L- and GUCA1AWT+p.R120L-injected zebrafish compared with the GUCA1AWT-injected group. (**h**–**j**) Robust Zpr-2 expression was detected in retinal frozen sections of 4-dpf larvae injected with GUCA1AWT (**h**), whereas Zpr-2 expression was diminished in the GUCA1Ap.R120L-injected (**i**) and GUCA1AWT+p.R120L-injected (**j**) groups. (**k**) Relative mRNA levels of RPE characteristic transcripts in the GUCA1Ap.R120L- and GUCA1AWT+p.

well-circumscribed atrophy of the RPE and choriocapillaris appear outside the fovea. In stage IV, well-defined chorioretinal atrophy affects the fovea and results in a markedly decreased visual acuity. There is no drusen and neovascular lesion in typical CACD. But CACD is a clinically heterogeneous disease and diverse in the age of onset, progression, and phenotype expression, even within one family [2, 17, 19]; in a large consanguineous Tunisian family, CACD segregated in an autosomal dominant trait. Among 21 affected family members, 6 patients had drusen deposits. The PRPH2 gene and the 17p13 locus were not associated with this phenomenon [6] The Arg142Trp mutation in PRPH2 is one of the factors predisposing to drusen development [7], while the Tyr141Cys mutation is susceptible to secondary development of choroidal neovascular membranes [20]. We reveal a novel GUCA1A mutation, p.R120L, in a family with variable maculopathies ranging from mild photoreceptor degeneration to CACD (Figs. 10.2 and 10.3). This means more attention should be attach on photoreceptor function examination on whom with undetectable physical sign in CACD family.

10.2.5 Molecular Biology

The molecular mechanism of CACD is still not clear now. The peripherin encoded by PRPH2 gene is a surface glycoprotein localized to the rim region of the disc membranes of both rod and cone photoreceptor, which functions as an adhesion molecule to play an essential role in the assembly, orientation, and structural stability of outer segment discs and to account for an increased turnover of instable membranous segments [17]. PRPH2 mutation may lead to less efficient clearing away of the instable membranous segments, and more OS debris accumulate beneath the retina, which is toxic to RPE. Another CACD causative gene GUCA1A, revealed in our study, encodes the guanylyl cyclase-activating protein1 (GCAP1) which is essential in the visual cycle and highly expressed in the outer segment (OS) layer of cones [21], which acts as a calcium sensor in the recovery of photoreceptors from photon capture by activation of the retinal guanylate cyclase 1 (retGC1). RetGC1 is a membrane-bound homodimer enzyme, which converts guanosine 5'-triphosphate (GTP) to cGMP, permitting reopening sodium- and voltage-gated calcium channels to recover from the dark state. The abnormal calcium sensitivity of the cyclase increases cGMP-gated dark current in the rod outer segments; overloading of calcium reshapes rod photoresponses and triggers photoreceptor death [22]. Both GUCA1A p.R120L mutations and GUCY2D p.V933A mutation can lead to CACD. The molecular mechanism of these two mutations functioning in CACD pathology needs to be further investigated.

10.2.6 Summary

Although CACD is a rare disease, the clinical and genetic diversity confuse us whether CACD represent a severe pattern of maculopathy or possess complete different pathogenic modes. Much work had to be done on the molecular mechanism.

10.3 Leber Congenital Amaurosis (LCA)

10.3.1 Introduction

Leber congenital amaurosis (LCA) is a hereditary early-onset congenital retinopathy that develops concomitantly with severe macular

Fig. 10.1 (continued) R120L-injected groups were decreased when compared with the GUCA1AWT-injected zebrafish. (**l**) No truncal vascular anomaly is revealed in transgenic (flk1: enhanced green fluorescent protein (EGFP)) zebrafish injected with GUCA1Ap.R120L. (**m**) Axial projections of confocal images from the two injected groups. Fluorescent intensity of EGFP is significantly reduced in the GUCA1Ap.R120L-injected group. (**n**) The fluorescent intensity of EGFP calculated by ImageJ in the two groups. Results were obtained from technical triplicates. Error bars represent the SE. Error bars represent the SD. *NS* not significant. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Scale bar = 20 mm

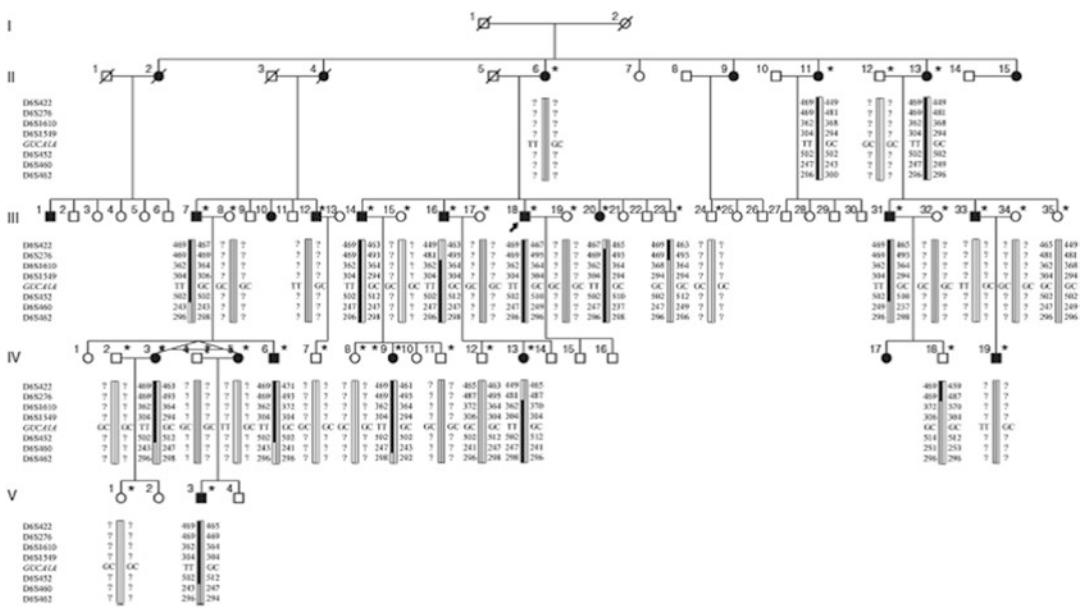

Fig. 10.2 Pedigree of family DC and haplotype reconstruction for the mapped region on chromosome 6 in family DC. Filled and open symbols represent affected and unaffected members, respectively. The proband is indicated by the black arrow. Haplotypes for tested short tandem repeat (STR) markers in the mapped region and those flanking it, and genotypes for GUCA1A c.359_360, are given for all participants. Black bars represent the ancestral haplotype associated with the disease. *Individuals from whom blood samples were collected. The mapped region flanked by markers D6S276 and D6S460 was shared by all patients and was absent in all unaffected members

degeneration [23].Clinical observations of LCA patients are heterogeneous, although LCA patients are usually born almost or completely blind. Thus, the parents of LCA patients typically notice their child's blindness within several months after birth [23]. Nearly 20% of blind school children have been diagnosed with LCA, and this disease represents a great burden on their quality of life [24]. Children with LCA are defined by a very low, or non-recordable, electroretinogram (ERG) during infancy, and the vision loss experienced in LCA patients is severe and progressive. The variety of clinical manifestations, combined with the onset of macular atrophy and pallor optic nerve head, are concomitant with the symptoms of LCA.

10.3.2 Clinical Features

The pedigree was recruited from Ningxia Eye Hospital, Ningxia People's Hospital. The parents were second cousins, and their child (II-6, in Fig. 10.4a) was diagnosed as blind with severe retinopathy at the age of 6 months. All of the members of this family were examined by fundus imaging and optical coherence tomography (OCT). Six members exhibited normal fundus and OCT images (Fig. 10.4c), while two of the six children were further diagnosed with LCA based on the presence of attenuated blood vessels and pallor fundus images (Fig. 10.4d). These observations were made in combination with the habitual oculodigital signs of photophobia and eye poking (Fig. 10.4e), clinical hallmarks of LCA. In addition, visual acuity tests performed for the affected individuals revealed no fixation of reflection and no following of movement. Except for the retinal dystrophy, both patients have hearing disorders and language barrier. But they had no polydactyly or renal cysts, the hallmarks of Bardet-Biedl syndrome (BBS) and ciliopathy.

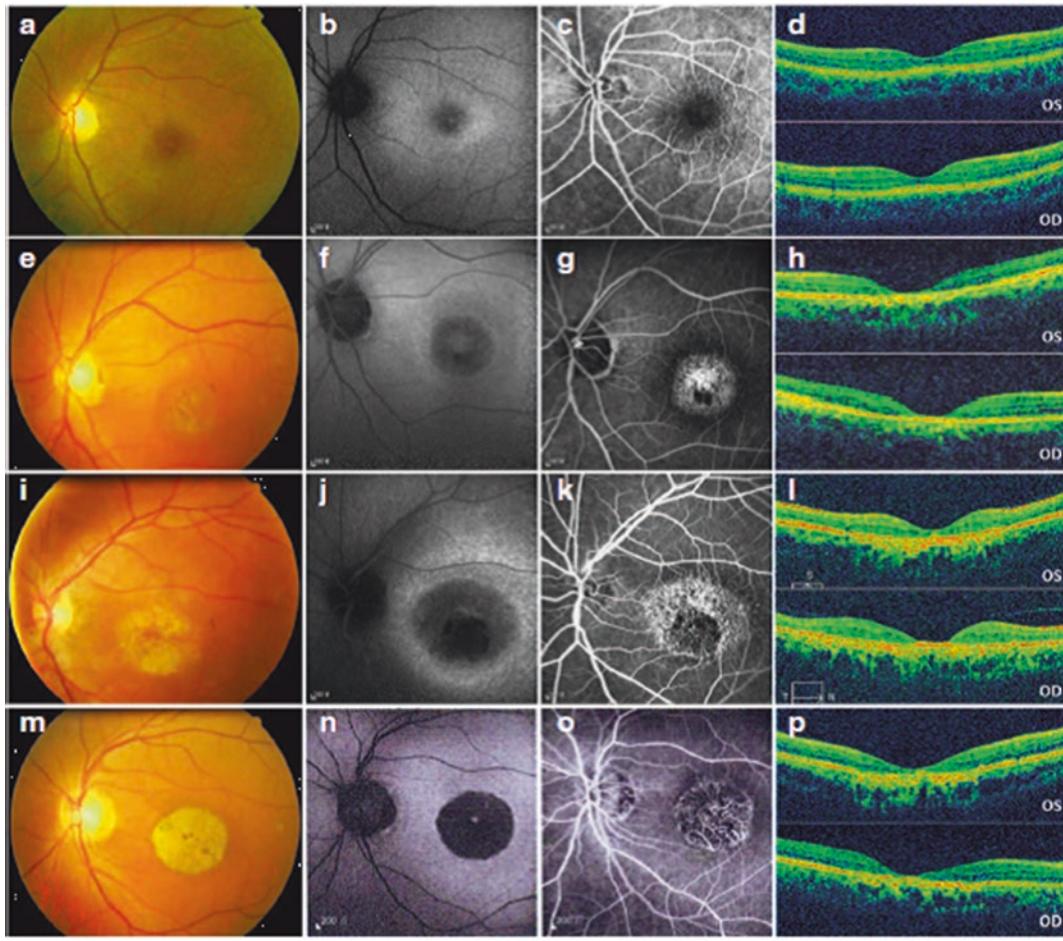

Fig. 10.3 Fundus photography, fundus autofluorescence (FAF), fundus fluorescein angiography (FFA), and optical coherence tomography (OCT) presentations for patients with different grades of maculopathy. (**a**–**d**) Patient DC-IV:6 with grade I maculopathy showed slight parafoveal hypopigmentation (a) with discrete increased FAF (**b**) and hyperfluorescent parafoveal changes on FFA (**c**). OCT revealed that the outer nuclear layer (ONL) and inner segment/outer segment (IS/OS) layer are slightly reduced in the fovea. The retinal pigment epithelium (RPE) layer was relatively normal (**d**). (**e**–**h**) Patient DC-III:16 with grade II maculopathy showed pigmental disturbance in the fundus (**e**) and speckled changes of increased fluorescence in the maculae, with a round area of hypofluorescence seen on FAF and FFA (**f**, **g**). OCT showed that the ONL and IS/OS layer were almost vanished in the fovea and its surrounding maculae, with no remarkable changes detected in the peripheral retina. Macular RPE was slightly changed (**h**). (**i**–**l**) In patient DC-III:14 with grade III maculopathy, a patch of circumscribed chorioretinal atrophy outside the central fovea is indicated on a fundus photograph. Slight hypopigmentation is found within this lesion (**i**). This area shows severely decreased to absent FAF (**j**), with choroidal vessels visible on FFA (**k**). OCT demonstrated that the ONL and IS/OS layer in the fovea had vanished. Macular RPE was moderately changed (**l**). (**m**–**p**) In patient DC-III:18 with grade IV maculopathy, the fundus photograph presents a well-demarcated area of chorioretinal atrophy involving the fovea (**m**), corresponding to an area of absent FAF (**n**). FFA shows a well-defined area of chorioretinal atrophy with a few large choroidal vessels visible (**o**). OCT shows a vanished ONL and IS/OS layer in the fovea. Macular RPE degeneration was the severest of all (**p**). OD = right eye; OS = left eye

10.3.3 Genetic Research

Further genetic analysis revealed that two novel compound heterozygous mutations, c.1198A > G andc.1547G > A, were present in this CCT2, and these represented unrivaled candidates in this LCA family (Fig. 10.4b).These mutations were located in exon 12 and exon 15 of the CCT2

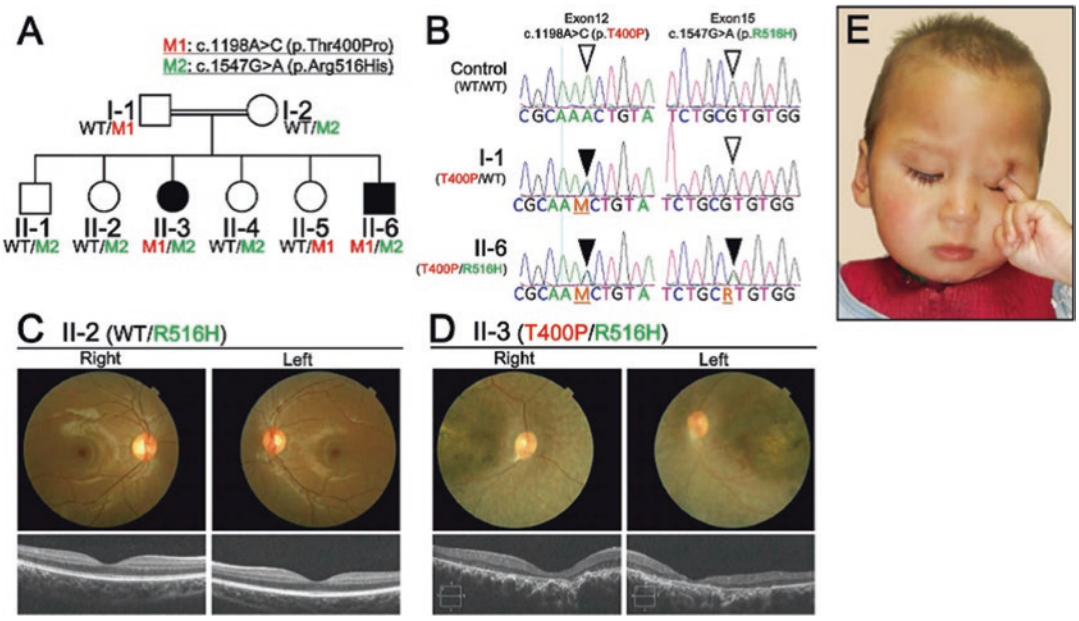

Fig. 10.4 Whole exome sequencing (WES) and clinical diagnosis. (**a**) Recessive patterning of a consanguineous LCA family was performed, and compound heterozygous mutations were identified. Whole exome sequencing was performed, and two point mutations in *CCT2* were found, c.1198A >C and c.1547G >A, which resulted in the following substitutions: p.Thr400Pro (p.T400P) and p. Arg516His (p.R516H), respectively.(**b**) *CCT2* gene mutations and corresponding amino acid substitutions in the CCTβ protein were identified. Point mutations in exon 12 (c.1198A >C) and exon 15 (c.1547G >A) resulted in the CCTβ missense changes, T400P and R516H, respectively. (**c**) Clinical evaluations of the non-affected individual, II-2. Upper panel: Color fundus photographs showed a normal retina. Lower panel: An OCT examination also showed a normal retina. (**d**) Clinical evaluations of the affected individual, II-3. Upper panel: Color fundus photographs showed severe degenerative changes. The optic disc was pale and the retinal blood vessels were attenuated. A maculopathy with chorioretinal atrophy and aggregation of the pigment were also observed. Lower panel: An OCT examination showed the thinning and disorganization of the retina. Both c.1198A >C (M1 in red)- and c.1547G >A (M2 in green)-carrying individuals (e.g., II-3 and II-6, respectively) exhibited retinal dystrophy and a macular degeneration phenotypes. (E) Facial and behavioral features of the oculodigital phenomenon (eye poking) and sunken eyes (exophthalmos) of the affected individual, II-6

mRNA transcript, respectively. The CCT chaperone machinery was found to be affected by the structurally instable T400P and R516H mutant proteins that exhibited an aberrant affinity to the CCTγ subunit adjacent to CCTβ. Moreover, the T400P- andR516H-carrying patient-derived T cells and iPSCs exhibited less proliferation. The T400P and R516H mutants also exhibited limited rescue effects on cell proliferation compared to wild-type CCTβ. Both subunits, CCTβ and CCTγ, were expressed in the cells in retinal ganglion cell layer and near the connecting cilium in the photoreceptor cells. In addition, levels of Gβ1, a major target of the CCT complex, and a critical protein in the phototransduction pathway, were decreased following the knockdown of CCT2 in a mouse photoreceptor cell line.

In conclusion, two novel compound heterozygous mutations in CCT2 were identified in association with a family affected by LCA. These mutations affected CCTβ-associated chaperone activity which has an essential role in retinal development and photoreceptor physiology.

10.4 Usher Syndrome

10.4.1 Introduction

Usher syndrome is a genetically heterogeneous disorder characterized by combined retinitis pigmentosa and sensorineural hearing loss. It is caused by disruption of protein components in the supramolecular Usher protein network. Usher syndrome has a prevalence ranging from 3.2 to 6.2 in 100 000 [25–27], and it can be classified into three major categories, namely, USH I, II, and III, depending on the age of onset and severity. Until now, 13 genes have been associated with Usher syndrome (10). However, mutations in these genes can only account for 70–80% of cases [28–30], suggesting additional disease-causing loci are yet to be identified.

10.4.2 Clinical Features

Patients in a consanguineous family were collected at the Ningxia Eye Hospital, Ningxia People's Hospital. The proband developed night blindness since early childhood. She also noticed hypochromatopsia and mild hearing loss since age 8. She suffered from visual field decrease and central vision loss since age 10. At her latest visit at age 35, her BCVAs dropped to 20/125 OD and 20/200 OS. Hypochromatopsia was confirmed by color vision test. Funduscopy features include attenuated vessels, waxy optic disc, and pigment deposits in the mid-peripheral retina (Fig. 10.5a, b). Macular region was also involved. Attenuated outer nucleus layer, retinal pigment epithelium (RPE), and loss of ellipsoid zone were suggested by OCT presentations (Fig. 10.5c, d). Consistent with the fundus photography, FFA also revealed aberrant vascular arcades and speckled changes of increased fluorescence in the mid-peripheral retina (Fig. 10.5e, f). Both scotopic and photopic ERG responses were abolished. She has an affected brother with similar ophthalmological findings as well as hearing loss. According to these abnormalities, both patients in this family were clinically diagnosed with atypical Usher syndrome phenotype.

10.4.3 Genetic Research

The homozygous variant that affects the canonical splicing acceptor site of exon 14 (c.1629-2A>G (p.G545Pfs*6)) of CEP78 was identified in both patients with Usher syndrome in this consanguineous family. It was not observed in any public control databases. Sanger sequencing also confirmed the genotype-phenotype cosegregation in this family (Fig. 10.6). Genetic screening in additional 71 unsolved patients with Usher syndrome did not identify additional cases with biallelic CEP78 mutations. We collected the patient blood and performed RT-PCR with Sanger sequencing. Finally, we confirmed that this variant indeed affects the pre-mRNA splicing in vivo and leads to frameshifts and premature stop codon.

In summary, our results identified a novel ciliary gene CEP78 involved in Usher syndrome. Further studies on additional cases and animal models would help to describe the CEP78-related phenotype more precisely and reveal the ciliary involvement in Usher protein network in photoreceptor cells and inner ear hair cells, thus pushing forward more efficient disease management and treatment for Usher syndrome.

10.5 Retinitis Pigmentosa

10.5.1 Introduction

Retinitis pigmentosa [RP (MIM 268000)], the most common form of inherited retinal dystrophies (IRDs), displays a prevalence ranging from 1/3500 to 1/5000 among different populations [31–33]. Clinically, photoreceptor degeneration and pigment migration are characteristics of RP, and symptoms include night blindness, a constricted visual field (VF), and sometimes eventual loss of central vision [34]. At the cellular level, rod photoreceptors and/or the retinal pigment epithelium (RPE) are affected at the initial stage of the disease, and cone photoreceptors may be involved at a later stage. At the molecular level, RP exhibits significant genetic heterogene-

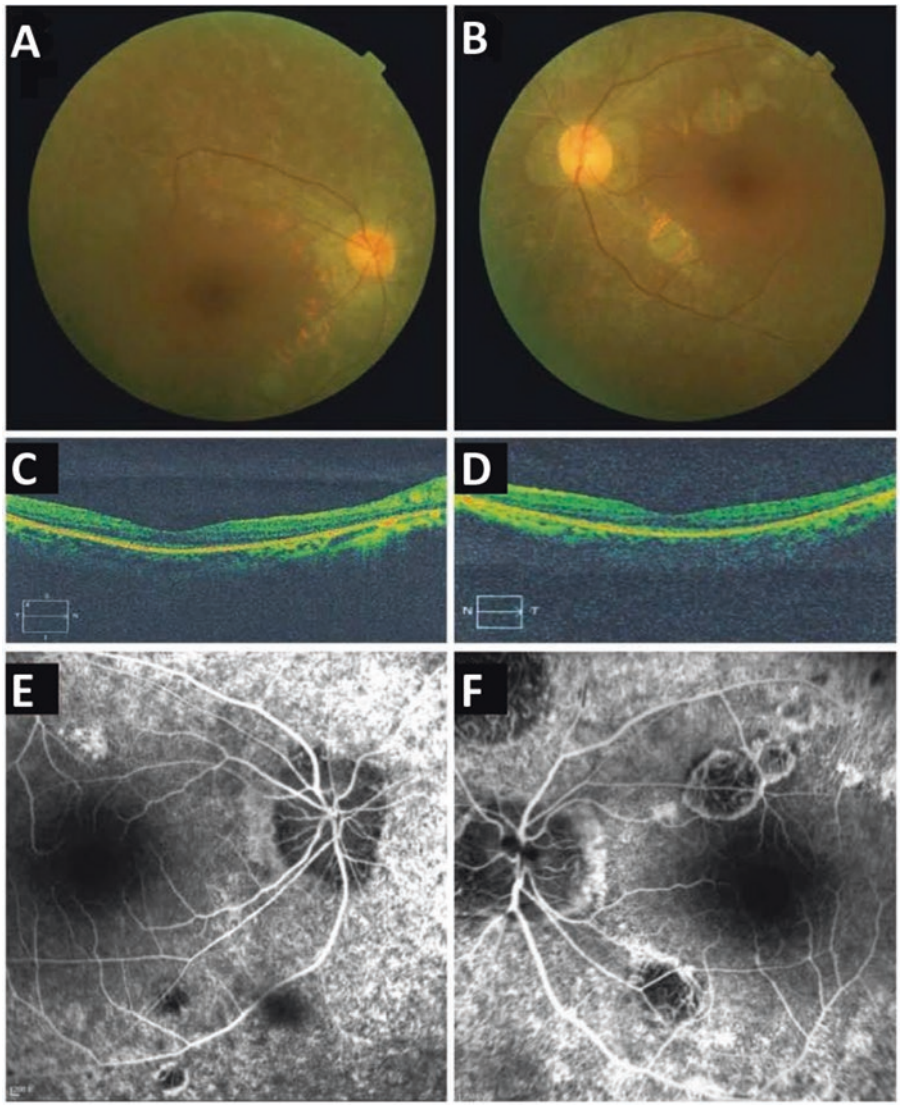

Fig. 10.5 Clinical manifestations of the patients with atypical Usher syndrome. Fundus images of OD (**a**) and OS (**b**). Optical coherence tomography images of OD (**c**) and OS (**d**). Fundus autofluorescence results of OD (**e**) and OS (**f**)

ity involving at least 87 disease-causing genes (RetNet).

10.5.2 Clinical Features

A three-generation family (AD01) with autosomal dominant retinitis pigmentosa (adRP) and a sporadic (S01) RP patient were recruited at the Ningxia Eye Hospital, Ningxia People's Hospital. The proband of Family AD01, AD01-II:3, was first referred to ophthalmic examination at the age of 15 for his poor night vision. After that, he developed VF restriction and impaired central vision. At the latest visit when he was 58 years old, he showed bilateral cataracts and a typical RP fundus, including retinal degeneration with macular involvement, waxy pallor of optic disc, narrowed vasculature, and peripheral pigment deposit (Fig. 10.7c versus e). Optical coherence tomography (OCT) revealed attenuated outer nuclear layer (ONL) and RPE at the maculae with complete loss

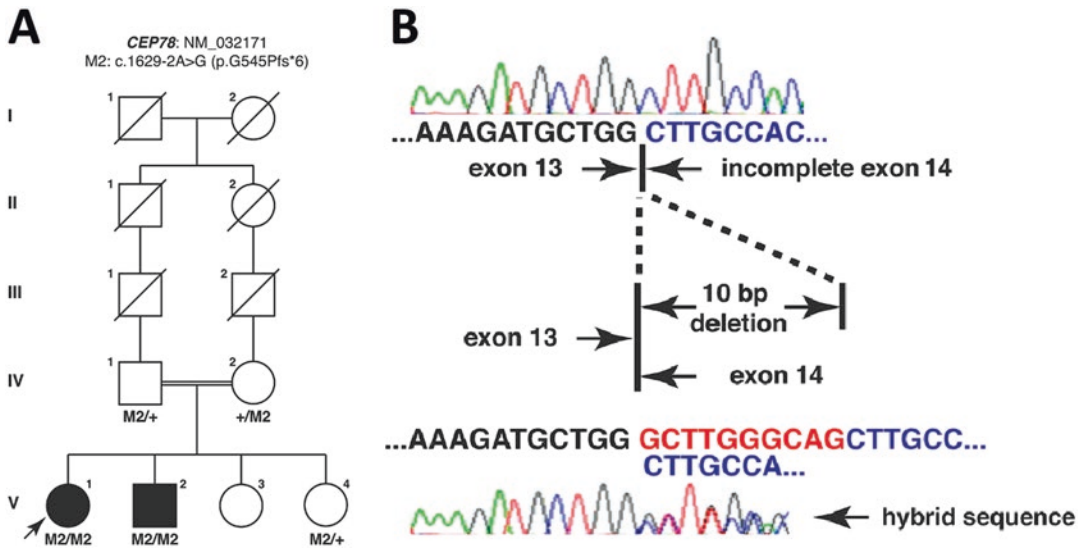

Fig. 10.6 Genetic findings in the patients with Usher syndrome. (**a**) CEP78 mutations were identified in two unrelated consanguineous families. The variants cosegregate with the disease phenotype. Arrows indicate probands. (**b**) RT-PCR and Sanger sequencing confirmed the splicing-altering effect of the variant c.1629-2A>G. In the probands' mRNA, a10 bp-long region in CEP78 exon 14 was deleted, while in the unaffected heterozygous carrier, wild-type/mutant hybrid sequence was observed

of outer/inner segments (OS/IS), all of which were consistent with his poor visual acuity (Fig. 10.7d versus f). Diffused loss of VF and diminished electroretinogram (ERG) responses were also observed. Patients AD01-II:1 and AD01-II:6 developed night blindness at 27 and 24 years of age, respectively. At the time of the last examination when they were 66 and 47 years of age, respectively, they showed phenotypes similar to those of the proband, including poor night vision, poor central visual acuity, severely reduced VF, typical RP fundus appearances, and nearly undetectable ERG responses. The individual AD01-III:4 was only 9 years old at the time of the last examination, whereas the ages at onset of disease in this family ranged from 15 to 27. Funduscopy and OCT examinations on AD01-III:4 detected no remarkable findings. ERG test revealed moderate defects including prolonged implicit time of b-wave in scotopic ERG and decreased amplitudes of a- and b-waves in photopic ERG compared with age-matched controls. We therefore diagnosed the AD01-III:4 as suspected RP. The Simplex patient, S01, developed night blindness at the age of 20. Constricted VF and decreased VA were noticed by 37 years of age. At a recent visit when he was 53 years old, he showed severely impaired VA, significantly restricted VF, undetectable ERG responses, and a typical RP fundus (Fig. 10.7g, h).

10.5.3 Genetic Research

We identified two heterozygous variants in PRPF4, including c.-114_-97del in a S01 RP patient and c.C944T (p.Pro315Leu), which cosegregate with disease phenotype in AD01 family with adRP (Fig. 10.8). Both variants were absent in 400 unrelated controls. The c.-114_-97del, predicted to affect two transcription factor binding sites, was shown to downregulate the promoter activity of PRPF4 by a luciferase assay and was associated with a significant reduction of PRPF4 expression in the blood cells of the patient. In fibroblasts from an affected individual with the p.Pro315Leu variant, the expression levels of several tri-snRNP components, including PRPF4 itself, were upregulated, with altered expression pattern of SC35, a spliceosome marker. The same alterations were also observed

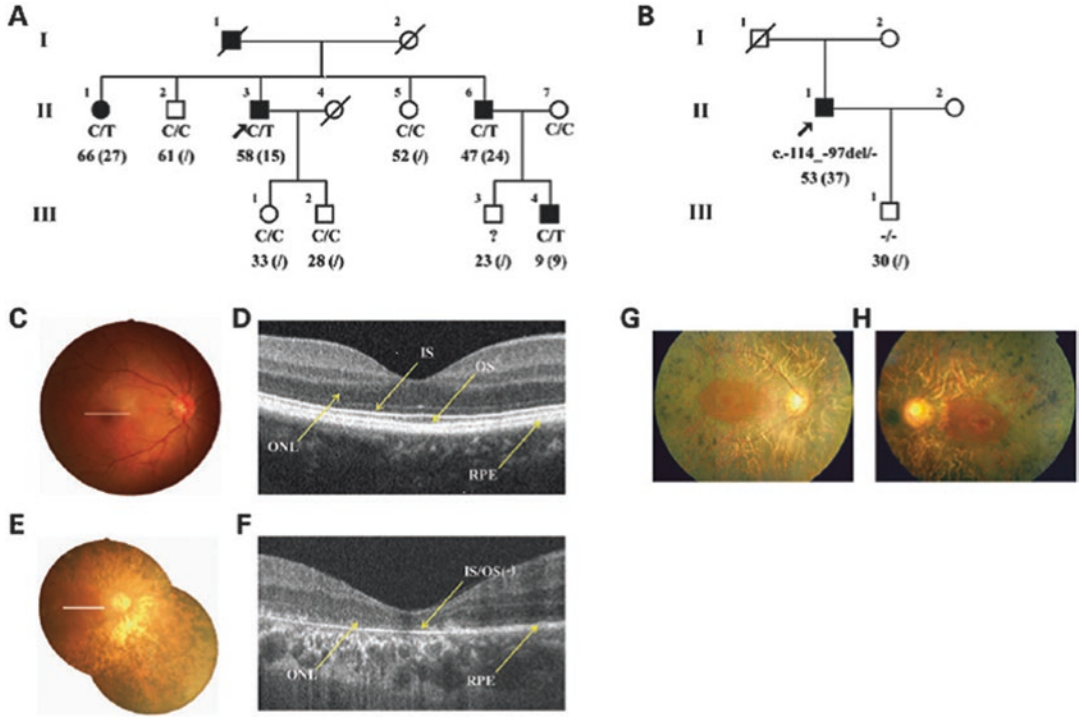

Fig. 10.7 Pedigrees and clinical evaluations of Family AD01 and Sporadic Case S01. (**a** and **b**) The pedigree of Family AD01 indicates a likely dominant inheritance of RP over three generations (**a**), while no other family members are affected within the pedigree of Sporadic Case S01 (**b**). The genotypes, age of final examination, and disease onset (inside parentheses) are given below the pedigree symbols. Black-filled and blank symbols represent affected and unaffected status, respectively. (**c** and **d**) Fundus and OCT examination of the right eye of the unaffected member (AD01-III:2) are provided as normal controls. (**e** and **f**) Right eye fundus of the proband (AD01-II:3) reveals typical RP degeneration (**e**), including a waxy optic disc, attenuated retinal vessels, and numerous bone spicule-like pigments. The white line indicates the layer for OCT examination (**f**), which demonstrates macular atrophy showing attenuated ONL and RPE with complete loss of OS and IS (denoted by yellow arrows). (**g** and **h**) The fundus of case S01 shows typical RP appearance for both eyes

in cells overexpressing hPrp4Pro315Leu, suggesting that they arose as a compensatory response to a compromised splicing mechanism caused by hPrp4 dysfunction. Further, overexpression of hPrp4Pro315Leu, but not hPrp4WT, triggered systemic deformities in wild-type zebrafish embryos with the retina primarily affected, and dramatically augmented death rates in morphant embryos, in which orthologous zebrafish prpf4 gene was silenced. We conclude that mutations of PRPF4 cause RP via haploinsufficiency and dominant-negative effects and establish PRPF4 as a new U4/U6-U5 snRNP component associated with ADRP.

10.6 Marfan Syndrome Combined with X-Linked Hypophosphatemia

10.6.1 Introduction

Marfan syndrome (MFS; MIM 154700), characterized by complicated manifestations in multiple organ systems with high degrees of clinical diversity, is one of the most common autosomal dominant inherited connective tissue diseases with a prevalence of 1 in 5,000[35]. Cardinal MFS features involve the ocular, skeletal, and cardiovascular systems, of which ectopia lentis and aortic aneurysm are given special clinical significance [36]. Excessive liner growth of the

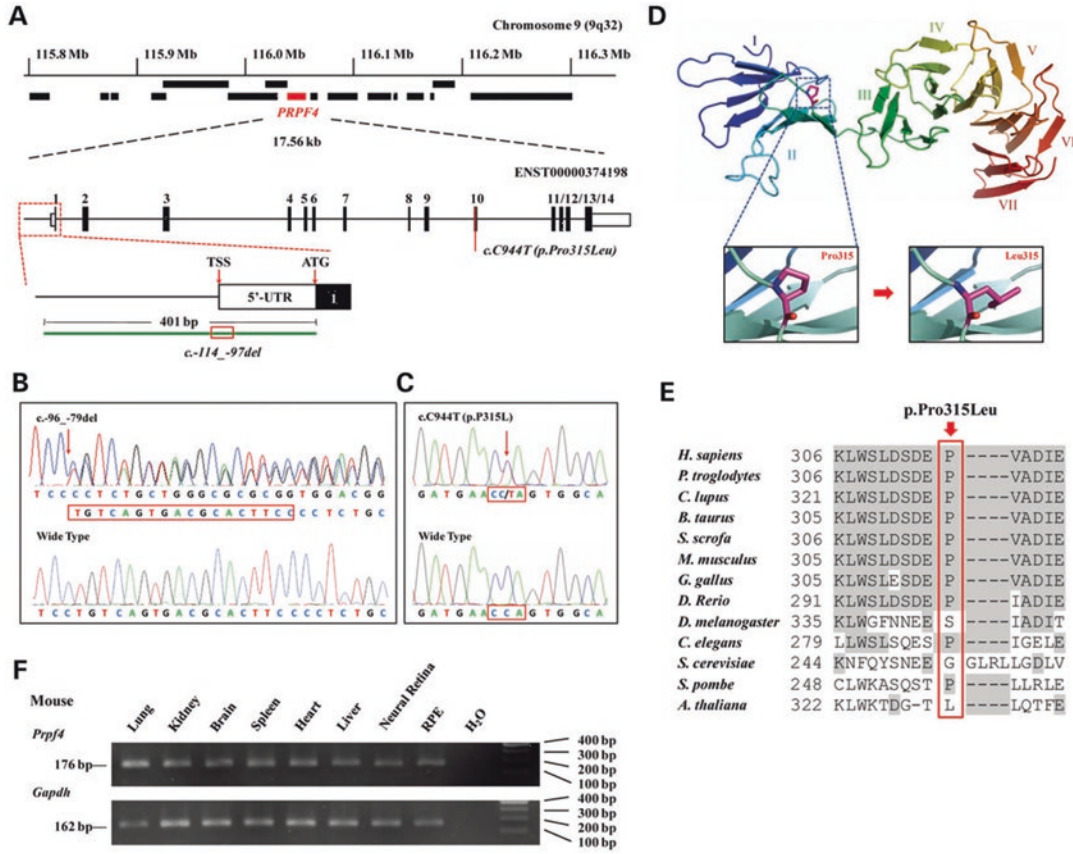

Fig. 10.8 Genetic analyses of variants identified in the PRPF4 gene and expression of Prpf4 in murine tissues. (**a**) PRPF4 gene (red-filled box) spanning 17.56 kb on chromosome 9q32 (upper panel) contains 14 exons (middle panel). The two identified heterozygous variants, c.-114_-97del and c.C944T (p.Pro315Leu), were indicated by a red box crossing the green line and a red line in the middle panel, respectively. The two red arrows denote the transcription start site (TSS) and the translation start site (ATG). (**b** and **c**) Sequencing chromatograms of the affected member from Family S01 (B) and AD01 (**c**) showing the c.-114_-97del and c.C944T substitution, respectively (top). WT sequences of the unaffected family members were shown at the bottom. (**d**) The top view illustrates the predicted structure of the WD-40 repeat domain of hPrp4 (residues 229–521), which comprises seven highly repeated blades. Each blade is indicated by different colors. Residue 315 is located in the second blade. A close view of residue 315 highlighting the WT (proline) and mutated (leucine) amino acids at the position is presented in the two bottom boxes. (**e**)Orthologous protein sequence alignment of PRPF4 from human (*Homo sapiens*), chimpanzees (*P. troglodytes*), dogs (*C. lupus*), cows (*B. taurus*), rats (*R. norvegicus*), chickens (*G. gallus*), zebrafish (*D. rerio*), fruit flies (*D. melanogaster*), roundworms (*C. elegans*), yeast (*S. cerevisiae* and *S. pombe*), and plant (*A. thaliana*). Conserved residues are shaded. The mutated residue 315 is boxed and indicated. (**f**) Expression of Prpf4 in multiple murine tissues including the lung, kidney, brain, spleen, heart, liver, neural retina, and retinal pigmented epithelium (RPE) is shown. A 176 bp PCR product of the murine Prpf4 (top panel) was detected in all tested tissues. PCR products (162 bp) of the murine Gapdh were analyzed in parallel as a loading control

long bones and joint laxity are hallmarks for the skeletal systems. X-linked hypophosphatemia (XLH;MIM 307800), presenting a prevalence of 1 in 20,000, is the most common form of familial hypophosphatemic rickets (FHR), which is a dominant disorder biochemically featured by hypophosphatemia caused by renal phosphate wasting with normal or low 1,25-dihydroxyvitamin D concentrations [37]. Low serum phosphate concentration and reduced tubular resorption of phosphate corrected for glomerular filtration rate (TmP/GFR) are characteristic for XLH [38].

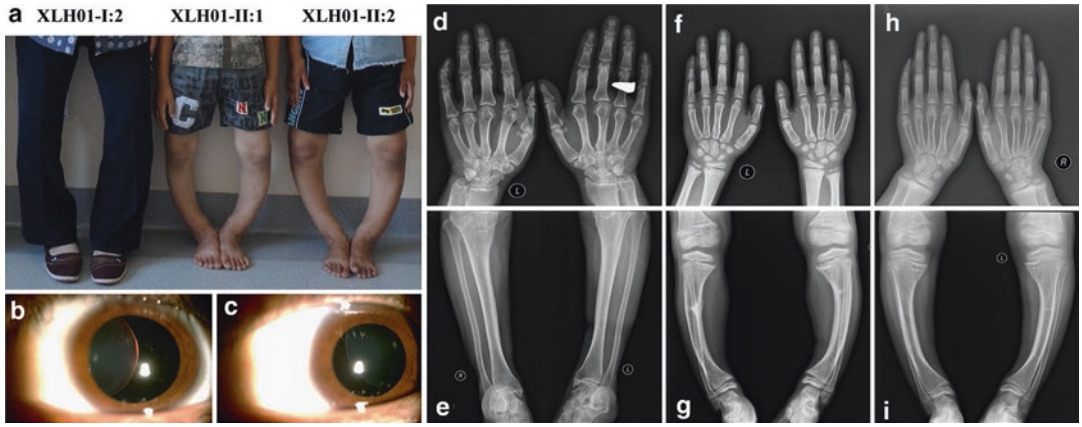

Fig. 10.9 Clinical presentation for family XLH01. (**a**) Medial bowing in the patients XLH01-I:2, XLH01-II:1, and XLH01-II:2. (**b**, **c**) Anterior segment photography indicates dislocation of lens toward nasal superior side in both eyes. (**D–i**) Radiographic findings reveal dolichostenomelia in the digits of patient XLH01-II:2 (**h**), but not in patient XLH01-I:2 (**d**) or XLH01-II:1 (**f**). Widening of both proximal and distal tibia metaphysis in patients XLH01-II:1(**f**, **g**) and XLH01-II:2 (**h**, **i**) are shown. Medial bowing is found in all three patients (**e**, **g**, **i**)

10.6.2 Clinical Features

In June 2011, the 8-year-old proband (XLH01-II:1) was first referred to our ophthalmic clinic for blurred vision over the past 2 years. She was born at term to a 34-year-old woman after an uneventful full-term pregnancy and delivery. Her best corrected visual acuities (BCVAs) reached 0.1 for the right eye and 0.25 for the left eye. Slit-lamp test revealed dislocation of lens toward nasal superior side and hippus in both eyes (Fig. 10.9b, c). Medical records included an atrioseptopexy at age 5 for her atrial septal defect (ASD). Other than the repaired ASD presentation, ultrasonic cardiogram (UCG) indicated left-to-right shunt flow through a ventricular septal defect (VSD) with the diameter of 4.50 mm. Slight mitral, tricuspid, and pulmonary regurgitations were also detected. Her calculated Z-score for aortic root was −0.54. Physical examination on our patient also showed complex skeletal problems. Her height was 117.0 cm [<−2standard deviation (SD)] and her weight was 30.0 kg(>+1 SD) on admission. Radiographic examinations showed widening of both proximal and distal tibia metaphysis with medial bowing, but dolichostenomelia in her digits was also revealed (Fig. 10.9h). Laboratory analyses indicated hypophosphatemia, elevated serum alkaline phosphatase (ALP) level, and reduced tubular resorption of phosphate corrected for glomerular filtration rate (TmP/GFR) in this patient. Serum calcium, intact parathyroid hormone (iPTH), 25-hydroxyvitamin D3, and 1,25-dihydroxyvitamin D3 were within the normal range. Her 41-year-old mother (XLH01-I:2) and 10-year-old brother (XLH01-II:2) had no remarkable ophthalmic or cardiac conditions, but showed similar skeletal abnormalities as demonstrated by physical and laboratorial tests with exception of dolichostenomelia in their digits (Fig. 10.9d, g). Her brother's height was 113.0 cm (<−3 SD). Her mother was 145 cm in height and had osteomalacia, severe ostealgia, and difficulties in walking, but no remarkable change in serum ALP level. The proband was there after treated with a surgical removal of the dislocated lens with implantation of an artificial intraocular lens and received amblyopia training after surgery. At the 3-year follow-up, her BCVAs improved to 0.4/0.5 (OD/OS). She also had follow-up visits in the cardiology and pediatric departments and was supplemented with elemental phosphorus and calcitriol. Her ALP level was back to normal at 2-year follow-up with no complication.

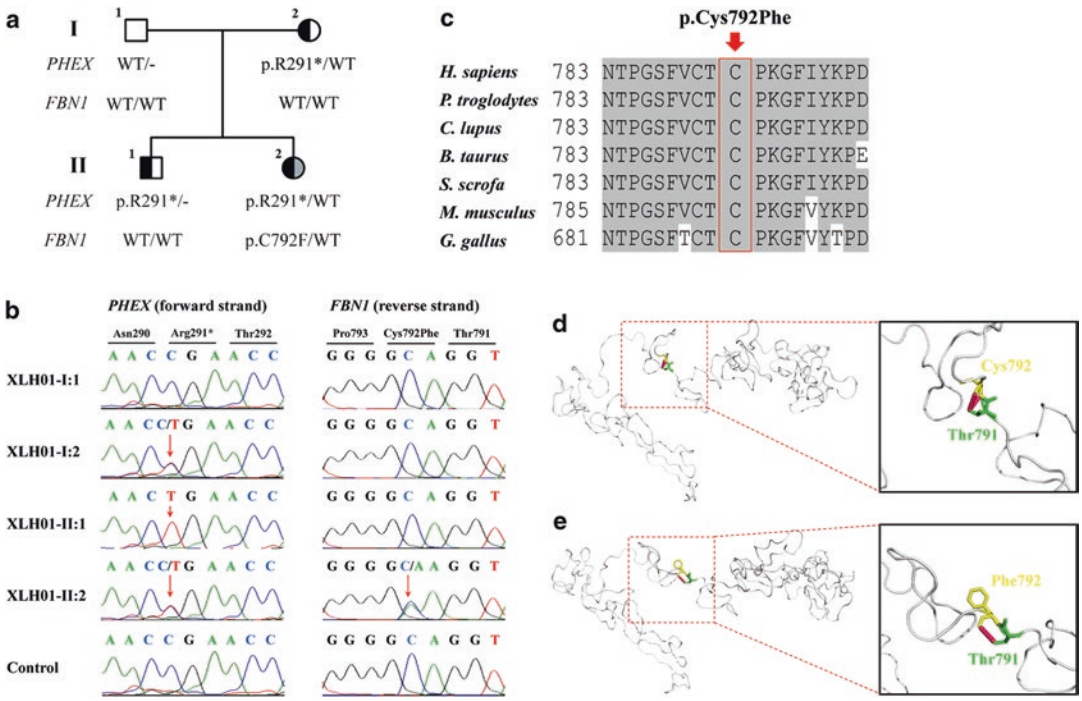

Fig. 10.10 PHEX and FBN1 mutations identified in the current family. (**a**). Pedigree of the included family. The PHEX and FBN1 genotypes for all included members were annotated. Black-filled, gray-filled, and blank symbols represent X-linked hypophosphatemia, Marfan syndrome, and unaffected status, respectively. (**b**). DNA sequencing profiles for the identified disease-causing mutations in the PHEX gene (left panel) and the FBN1 gene (right panel). (**c**) Orthologous protein sequence alignment of PHEX from seven species. Conserved residues are shaded. The mutated residue 792 is boxed and indicated. (**d**–**e**) Structural modeling of the wild-type and mutant fibrillin-1. One hydrogen bond in the wide-type protein was eliminated due to the substitution from cysteine to phenylalanine at residue 792

10.6.3 Genetic Research

We performed whole exome sequencing (WES) on patients XLH01-I:2, XLH01-II:1, and XLH01-II:2. A recurrent heterozygous/hemizygous mutation in the PHEX gene (c.871C>T [NM_000444]; p.R291* [NP_000435]) was identified in all three affected individuals. This mutation was previously reported in a patient with XLH. In addition, a de novo heterozygous mutation in the FBN1 gene (c.2375G>T [NM_000138]) was only called in the affected girl (Fig. 10.10a, b). This mutation would cause the amino acid change from the hydrophilic cysteine to the hydrophobic phenylalanine at residue 792 (p.C792F [NP_000129]). Residue Cys792 was predicted to be highly conserved among multiple species. Crystal structural modeling of the wild-type and mutant fibrillin-1 (residues 584–950) was constructed on the basis of low-density lipoprotein receptor (protein data bank [PDB] ID: 1N7D) with the sequence similarity of 0.38 (Fig. 10.10d, e). One hydrogen bond between residue792 and Thr791 was eliminated due to the substitution from cysteine to phenylalanine, which significantly altered its tertiary structure and would further change relevant protein properties.

10.7 Autosomal Recessive Bestrophinopathy(ARB)

10.7.1 Introduction

Autosomal recessive bestrophinopathy (ARB) was characterized by macular degeneration with

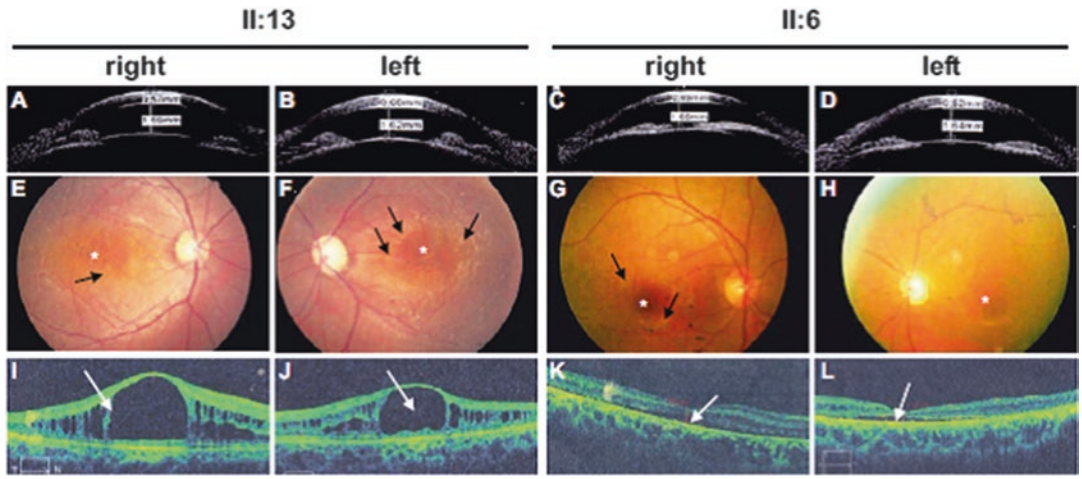

Fig. 10.11 Clinical evaluations of two patients, II:13 (upper left) and II:6 (upper right). (**a**–**d**), Ultrasound biomicroscopy revealed shallow anterior chamber and angle closure. (**e**–**h**), Color funduscopy indicated macular degeneration with yellowish-white deposits (black arrows) and retinal pigment epithelium (RPE) pigmentary atrophy (white asterisk) in both patients. Optic disc pale with increased cup-to-disc ratio (CDR) was observed in both eyes of II:13(**e**, **f**) and left eye of II:6(**h**). (**i**–**l**), Optical coherence tomography (OCT) examination demonstrated dramatic bilateral macular cystoid edema of II:13(**i**, **j**) and moderate accumulation of fluid in the subretinal space with RPE detachment at the fovea in II:6(**k**, **l**). White arrows indicate fluid accumulation

scattered punctate deposits and accumulation of fluid within and/or beneath the neurosensory retina [39]. Homozygous *BEST1* mutations were associated with ARB.

10.7.2 Clinical Features

We studied a consanguineous Chinese family (Fig. 10.12a) with five individuals possessing complex ocular phenotypes. Individuals I:1 and I:2 are first-degree cousins and had no history of eye diseases at any point in their lives. Four patients (II:6, II:12, II:13, and III:1) manifested angle-closure glaucoma (ACG) as demonstrated by dark-room gonioscopy, ultrasound biomicroscopy, increased intraocular pressure, and increased cup-to-disc ratio. Consistent with ACG, all four patients had moderately shortened axial lengths with mild hyperopia. They also had bilateral cortical cataracts, which by patient report developed before the age of 20 years (Fig. 10.11). Fundus examination revealed in all four patients' characteristic features of ARB including retinal pigment epithelium (RPE) disturbances and scattered punctate yellow-whitish fleck deposits surrounding maculae and accumulation of fluid in the subretinal and/or intraretinal space at the fovea (Fig. 10.11). Moreover, a significant reduction in the EOG light rise with moderately decreased ERG responses was detected in all patients, implying a primary insult to the RPE. Patient II:5 was unavailable for our clinical reevaluation, but her medical records indicated the development of bilateral ACG, premature cataract, and maculopathy by 40 years of age. A total of 13 other family members were examined and had no remarkable ophthalmic findings.

10.7.3 Genetic Findings

By next-generation sequencing and Sanger sequencing, only one variant, c.752G_A (p.C251Y) in *BEST1*, was identified as a putative pathogenic mutation. Sanger sequencing confirmed the presence of this variant in all five patients in the homozygous state and in ten unaffected family members in the heterozygous state (Fig. 10.12a). Of note, patient III:1 is homozygous for the mutation, suggesting that his unaffected father, who is not known to be related to the family, also harbors this variant, presumably

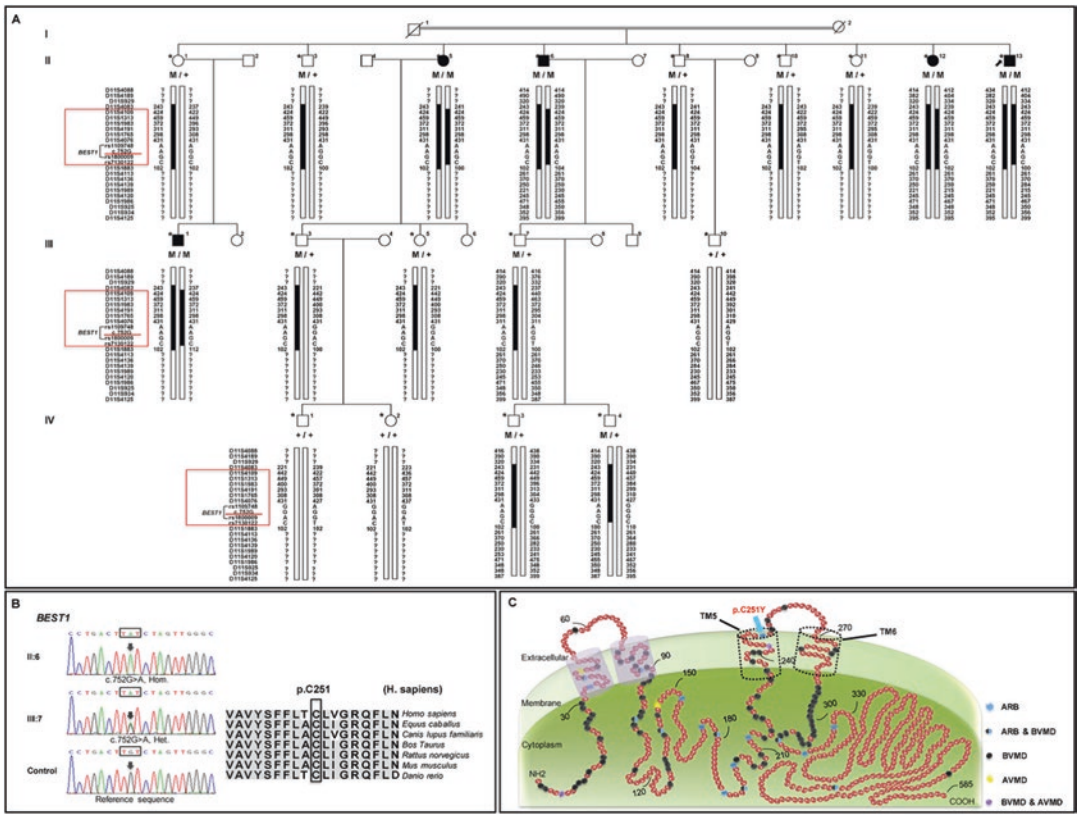

Fig. 10.12 Analysis of mutation BEST1 c.752 G_A (p. C251Y) and haplotype reconstruction for the homozygous region on chromosome 11 in the family. (**a**) Haplotype of the family. M _ BEST1 c.752 G_A. Affected and unaffected members are indicated by filled and open symbols, respectively. The black arrow indicates the proband. Genotypes are given for all participants (*). The black-filled bars represent ancestral haplotype associated with the disease. The homozygous region (denoted by red boxes) flanking by markers D11S4109 and rs7130122 was shared by all patients. The site of BEST1 c.752 G_A was indicated by red lines underneath. (**b**) Sanger sequencing showing homozygous and heterozygous c.752 G_A mutation in II:6 (patient) and his son, III:7 (unaffected), respectively. Reference sequences are given at the bottom. Het, heterozygous; Hom., homozygous. Conservation analysis of residue p.C251 (boxed) of bestrophin-1 across seven species was shown on the right panel. (**c**) Schematic structure of bestrophin-1.5. Known mutations are indicated by different symbols according to diagnoses. The fifth and sixth transmembrane-spanning helices of bestrophin-1 (TM5 and TM6) are denoted by dashed lines. Note that p.C251Y is the only ARB-associated mutation located in the TM5 domain

owing to a founder effect. This variant is absent in the 3 remaining unaffected family members tested and in 100 unrelated controls, is evolutionarily conserved (Fig. 10.12b), and is predicted to be functionally highly deleterious by the bioinformatic programs PolyPhen-2 (score _ 1) and SIFT (score _ 0). p.C251Y is located in the fifth transmembrane-spanning helix (TM5), 5 very close to the extracellular surface (Fig. 10.12c).

The cosegregation of p.C251Y with a coherent ocular phenotype in all five patients strongly suggests a new constellation of traits associated with *BEST1* homozygous mutations: ARB, ACG, hyperopia, and cataracts. Our extensive genetic analyses likely rule out other genetic components in this family.

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

Compliance with Ethical Requirements In our study, all procedures followed were in accordance with the ethical standards of the Committee of Ningxia People's Hospital on human experimentation and with the Helsinki Declaration of 1975, as revised in 2010.

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11 Ophthalmic Genetics in India: From Tentative Beginnings in the 1980's to Major Achievements in the Twenty-First Century

Govindasamy Kumaramanickavel
and M. J. Denton

Abstract

The journey to map autosomal recessive retinitis pigmentosa (RP) genes both syndromic and non-syndromic started in mid-1990 in India. The potential of consanguineous, large sibship families was exploited with overwhelming results particularly using homozygosity mapping methodology. The project came to standstill due to limited funding and complicated logistics to work in field in India. Even though the struggle started with multiple barriers and challenges, it was one of the most successful stories that a team of ophthalmologists and vision scientists in India and New Zealand enjoyed. These sincere efforts have led today towards promising gene therapy. This is a narrative story of those yesteryears.

Keywords

Autosomal recessive · Consanguinity · Linkage analysis · Ophthalmic genetics · Retinitis pigmentosa

11.1 Introduction

During the mid- to late 1980s, while one of us M.J. Denton (MJD) was establishing a gene mapping lab in the Prince of Wales Hospital (POW) in Sydney New South Wales, the other G. Kumaramanickavel (GK) was studying and collecting RP families in Chennai. Our eventual meeting in early 1989 proved to be a watershed in Asian or more specifically south Asian ophthalmic genetics as it initiated the first concerted effort to identify the genes responsible for inherited retinal diseases in India and indeed in Asia and led to some major advances in our understanding of genetic retinal diseases. Here we describe how our collaboration started in the late 1980s and how it matured over the next two decades into an important productive ophthalmic research project. We also acknowledge a number of genetic and ophthalmic researchers whose assistance was vital to the overall success of the project.

11.2 Beginnings: The Prince of Wales Hospital

In 1984 the head of pathology at the POW Professor Sidney Bell asked MJD to set up the gene mapping lab in the pathology department.

G. Kumaramanickavel (✉)
Narayana Nethralaya, Bangalore, India

M. J. Denton
University of Otago, Dunedin, New Zealand

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The aim of the lab was (1) to identify the chromosomal location of human disease genes by applying the then revolutionary technique of Southern blotting in conjunction with radiolabelled probes to detect 'marker RFLPs' linked to the disease genes which was at the time seen to be the first step towards the actual identification of the disease gene itself and (2) to carry out genetic diagnoses on individuals and families in which disease genes were segregating.

MJD had the good fortune to be working in a medical centre with a world-class collection of X-linked retinitis pigmentosa (xlRP) pedigrees, which had been collected and documented by Dr. Francis Halliday over several decades in the Sydney area of Australia. At the time (early 1984), none of the genes responsible for any form of RP including the X-linked form had been identified nor was the chromosomal location for any RP gene known even approximately. Intriguingly it was assumed that there would be one gene for each of the three forms of RP, xlRP and the dominant (adRP) and recessive forms (arRP). Given the wonderful collection of families, it seemed very natural to commence a project aimed at identifying the gene responsible for xlRP.

However shortly after the Sydney lab was operational, Professor Shomi Bhattacharya who was at the time stationed at the MRC unit in Edinburgh headed by Ed Southern [who was a leading researcher in the nascent field of human genomics and who had developed the key technique of Southern blotting which everyone at that time used to detect restriction fragment length polymorphisms (RFLPs) linked to disease loci] published a landmark paper in *Nature* reporting that the location of the xlRP locus was closely linked to an X-linked marker locus on the short arm of the X chromosome called LI28 [1]. Assuming that there would be only one xlRP gene [something that nearly everybody assumed at the time] and assuming that it would be closely linked to L128, MJD geared up to confirm the location by linkage analysis of the Halliday's families and to start a major genetic diagnostic service for XLRP patients, offering prenatal diagnosis and carrier detection.

However to MJD's surprise [and everybody's working in the field], it turned out that the genetics of XLRP was far more complex than originally thought and might have been inferred from Shomi's initial paper. It was apparent from the very first linkage analysis studies that in many of Halliday's families the xlRP gene was some distance from the marker L128 and subsequent linkage studies of all Halliday's families with a series of X-linked probes to determine the genetic distance from L128 confirmed that the commonest xlRP locus was not closely linked to L128. Because many of Halliday's pedigrees were large multigenerational families and excellent for gene mapping purposes, the xlRP work at the Prince of Wales led to important advances in knowledge of the genetics of X-linked RP including the most accurate location of the most common form of xlRP [2]at the time and the first clear evidence from analyses carried out in conjunction with Professor Andreas Gal (who was then at the Institute of Human Genetics in Bonn, West Germany) that the disease was genetically heterogeneous [3]. This fact was increasingly confirmed by subsequent work by many [4].

11.3 Autosomal Recessive RP, India's Potential

It was the early realization by MJD that xlRP was heterogeneous and the likelihood that the other forms of RP including *the commonest form of the disease*, arRP, might also be heterogeneous that led MJD to first consider in the late 1980s the possibility of studying Indian families with arRP. In Australia, families ideal for locating and identifying recessive disease genes, i.e. consanguineous and with large sibships, are very rare, but such families are relatively common in south Asia. If the disease was to be heterogeneous as MJD suspected, then combining linkage information from several small families might not be feasible. The best strategy would be to study individual pedigrees with sufficient members affected to provide a LOD ['logarithm of the odds'] score of 3, indicative of a close relationship between a marker locus and a disease gene. And India is

probably the best source of such families in the world thought MJD.

MJD was encouraged to visit India regarding the prospects of initiating a recessive gene mapping project by the Australian of the year Professor Fred Hollows [5] who was director of the Ophthalmology Department at the POW and who had devoted considerable energy and was well known for his efforts in organizing eye camps in various countries including Eritrea and Nepal.

11.4 Beginnings: Chennai

While MJD was building a gene mapping research group in Sydney and starting to contemplate the possibility of studying Indian families to locate arRP genes, GKM [who gained his MBBS degree from Madras Medical College in 1982] was completing his MD in Physiology [at Madras Medical College, awarded his MD in 1986] and had started his postgraduate studies of inherited retinal diseases and was collecting and ascertaining RP families in Tamil Nadu. MJD first learnt of GKM's work and pedigree collection through a fortuitous meeting with another genetics researcher at the University of New South Wales, Dr. Edward Max Nicholls, who had a long time interest in Indian genetics and happened to meet GKM in 1987 in Chennai, and on returning to Sydney, he informed MJD of GKM's work on arRP and his growing pedigree collection and suggested that MJD contact him to raise the possibility of jointly working with him on the genetics of arRP.

Having made initial contact by mail and phone in 1988 with GKM and having received a copy of GKMs pedigrees and agreed tentatively to a meeting in early 1989 MJD made an application for funding to the British RP Society [during 1988] to initiate an Indian recessive gene mapping project. The submission was successful, and with the funds provided, MJD made his first visit to India in January 1989 and spent some time with GKM in Chennai discussing the prospects for commencing a major arRP gene mapping project which would be the first systematic attempt in India and probably anywhere in Asia to map recessive disease genes. During the visit GKM introduced MJD to his Professor in Madras Medical College and to a prominent ophthalmologist in Chennai, Prof NS Sundaram, who were very supportive. In discussion between us [MJD and GKM], it was agreed that the key to the project success would be to collect a set of large multigenerational arRP pedigrees each with multiple affected and sufficiently large to map the disease gene in that family.

Later in 1989, MJD revisited India to meet up with GKM to initiate the building of a collaborative network of researchers, to assist with the collection of the arRP pedigrees. Fortuitously MJD had an Indian student in his Sydney lab, Aarti Chand, who was completing Honours BSc at the University of NSW, who had contacts in Mumbai and at AIIMS in Delhi and was keen to assist in any way she could. In Mumbai Aarti introduced MJD to Dr. DK Gahlot who had a large RP clinic in the city and who had a large number of arRP families and agreed to collaborate in the study. Aarti also arranged a meeting with members of the Parsi community in which arRP was quite common as well as an investigatory visit to Ahmedabad. In Delhi Aarti introduced MJD to Prof I C Verma [Professor of Human Genetics at AIIMs] who was very supportive and organized a talk at AIIMs for MJD to stimulate interest in the potential of India for recessive gene mapping. After Mumbai and Delhi, Aarti and MJD visited GKM in Chennai, and on GKM's suggestion we visited the Sankara Nethralaya, to meet the director Dr. SS Badrinath to request his collaboration and approval of the project which he generously granted.

This was an important milestone in the initiation of the project as Nethralaya was one of the most, perhaps at the time and even now, prestigious and advanced ophthalmic hospitals in India, and families collected through this institution would be very thoroughly ascertained and clinically assessed. Dr. Badrinath introduced us to Drs Mary and Chandran Abrahams, retina specialist couples, who showed us some of the Nethralaya collection of RP pedigrees. After Nethralaya obtained approval from the Indian

Council of Medical Research, Government of India, to conduct the study in collaboration with MJD and GKM, MJD took up an appointment as a Senior Medical Research Fellow at the University of Otago in Dunedin, NZ, in the early 1990s with the specific purpose of setting up a major recessive RP research project with GKM based in the Biochemistry Department of Otago University.

11.5 Collecting the Pedigrees

During the next 2 years [approximately early 1990–late 1991] after having established collaboration with Nethralaya in Chennai and after MJD had moved to the University of Otago, we (MJD and GKM) made a major effort to interest leading ophthalmologists in south India to join the project. We made many visits to various important ophthalmic centres, and some of the collaborators who agreed to join the project included Dr. Rama Murthy in Bangalore [whom we contacted at Dr. Badrinath's recommendation]. At the 1991 All India Ophthalmic Society (AIOS) meet in Bangalore, MJD had gone to meet up with Dr. K Rama Murthy. He also met Dr. Joseph Mani, an ophthalmologist stationed in Kerala who invited us to visit the Little Flower Hospital in Angamaly (Kerala) to investigate the potential of Kerala to collect arRP pedigrees. Taking up his invite we visited Kerala in late 1991, where he met up again with Dr. Mani and met Dr. Tony Fernadez and Dr. JK Mukkadan who was the research director at the Little Flower Hospital at the time. Visiting Kerala was quite fortuitous as it turned out that Dr. Mukkadan had a very impressive collection of arRP pedigrees. Several visits were also made to another leading ophthalmic centre, the LV Prasad Eye Institute in Hyderabad, where the director G N Rao also gave his blessing to the project.

During the next few years of the project approximately from 1991 to 1993, we [MJD and GKM] set out energetically to ascertain and collect blood specimens from patients and families provided by the collaborating centres. As we were working on a shoestring budget, this necessitated in many instances arduous bus or taxi journeys to and from major urban centres and from urban centres to remote villages. We [MJD and GKM] did all the final pedigree ascertainment in the patient's home, ourselves, and all blood specimens were again taken by ourselves. Pedigrees collected between 1990 and 1993 included the first two families in which mutations in the retinal epithelium were shown to cause arRP. One of these was the family with the celebrated mutation in the RPE65 gene provided by both Sankara Nethralaya and Rama Murthy, which we [MJD and GKM] collected in Chennai and suburban Bangalore, respectively. The RPE65 gene was used in the first successful application of gene therapy in the field of ophthalmic genetics [see below].

Those families that had not been clinically examined in one of the collaborating centres had to be brought into one of the collaborating clinics for a thorough ophthalmological workup which included in most case ERGs. Virtually all the pedigrees included in the project save one or two Parsi families from Mumbai were collected in south India. By late 1993 the collection consisted of approximately 50 inbred arRP families each with multiple affected and each large enough to provide independent evidence (by linkage) of the location of the arRP gene. *This collection was certainly at the time one of the largest if not the largest single collections of large inbred arRP pedigrees with multiple affected, suitable for gene mapping purposes anywhere in the world.*

A problem we had to surmount was collection of blood specimens. We chose to collect blood samples from the families on newborn blood spot cards, which improved the compliance. Although this complicated the analysis of the DNA as it had to be extracted from the blood spot and the quality was not as good as DNA extracted from whole blood, however it simplified the actual collection as carrying a set of blood cards was a lot simpler in Indian conditions than carrying whole blood specimens. Logistically it was easier in many ways, which finally, looking back, turned out to be one of the key points for the success of the project.

The collection was overall a quite arduous process. Conditions were primitive in many places of India in the early 1990s before the transformative economic development of the past 25 years, and we had many memorable experiences travelling round south India, some amusing and others not too funny. On one occasion just as we were about to place a needle in the arm of a patient near Chennai, there was a power outage, and the venesection had to be continued by torch light. But there were lighter moments. On one occasion MJD was returning from Kerala to Chennai by train on the eve of Diwali when he was awoken in Coimbatore, just after having crossed the Kerala-Tamil Nadu border to an unbroken cacophony of explosions what he assumed must be a terrorist attack having not previously experienced the intensity of the fireworks displays associated with the celebration of Diwali in Tamil Nadu. Sleeping from Coimbatore to Chennai thereafter was simply impossible. On another occasion in Agra, Aarti having asked the cost of a shoe shine assuming it would be for both shoes was surprised to find that the quoted price was for one shoe only! On another occasion having asked the driver of a cab in Kerala why he was driving round bends on the wrong side of the road, he retorted 'not to worry it's my lucky day!' Another tout in Kerala requested some money for reciting one to ten backwards in Italian. On another occasion while we were on route to Vellore from Bangaluru, we noticed a VW beetle several metres above the ground in the branches of a tree. How it got there we had no idea and assumed that it had parked on top of a small sapling years ago and gradually been carried aloft by the growing tree. Another amusing scenario occurred in the *Bangalore Club* where MJD stayed many times as a guest of Dr. Rama Murthy. Due to repeated bouts of gastric upset, mostly he ordered toast and black tea for breakfast; a regular waiter familiar with his 'tummy upsets' would greet him in the morning, 'toast and tea as usual sir!'

11.6 DNA Analysis, Linkage and the Otago Lab

In 1992 MJD and GKM applied successfully to the NZ Health Research Council for support including a fellowship for GKM to spend time in NZ. And in 1993 The US Foundation Fighting Blindness provided a large research grant to provide further support for the project, for the collection of additional pedigrees in India and for the setting up of a gene mapping laboratory in Otago.

And the following year, the US NIH provided another grant to collect and study a set of arRP families collected in collaboration with the LV Prasad in Hyderabad which were to be analysed in the gene mapping lab at the National Eye Institute, NIH, headed by Fielding Hejtmancik.

Because of the size of the pedigree collection and the resources needed in the early 1990s to carry out linkage analyses on so many pedigrees, it made sense for us to collaborate with other large genetics laboratories such as Fielding's at the NIH. Because MJD had worked productively with Professor Andreas Gal on xlRP, it was agreed to send samples from many of the families for linkage studies to his lab in the Institute of Human Genetics in the University of Bonn. [Professor Gal is currently Professor Emeritus, former Director of the Institute of Human Genetics at the University Medical Center Hamburg-Eppendorf.]

The gene mapping lab in Otago was headed for most of the 1990s by Dr. Marion Maw who was responsible for carrying out the linkage analyses in Otago and supervising DNA analyses in the lab. The setup was small, consisting for most of the period of Marion and two research assistants plus one or two honours students. GKM arrived on his first visit to NZ in February 92 and was a NZ Health Research Council Fellow till September 97. During this period each year GKM spent several months in the Otago lab and several months with MJD in continuing pedigree

collection in India. During this period in addition to arRP pedigrees, some pedigrees with other genetic eye diseases were also collected, including one with Oguchi disease and one with blepharophimosis.

Between 1992 and 2000, molecular genetic studies of these Indian families in Otago and in Andreas Gal's lab resulted in a number of important results including:

1. Identification of the first case of arRP caused by a single amino acid change in rhodopsin [6].
2. Identification of the gene on chromosome 2 responsible for the form of hereditary night blindness known as Oguchi disease [7].
3. Identification of a new arRP gene on chromosome 15 encoding for cellular retinaldehyde-binding protein, a protein involved in the visual cycle in the RPE [8].
4. The finding that mutations in RPE65, a gene expressed in the retinal epithelium cell which encodes a protein involved in the visual cycle, are responsible for arRP [9].
5. Identification of a new arRP locus on chromosome 16p12.1–p12.3 [10].
6. Location of the gene responsible for blepharophimosis syndrome shown to be on chromosome 7p [11].
7. The demonstration that mutations in the gene (PDE6A) encoding the alpha subunit of cGMP phosphodiesterase (PDE) are probably a rare cause of arRP in Indian families [12].
8. Evidence of a new arRP locus on chromosome 2 [9].
9. Evidence that mutations in the prominin (mouse)-like 1 gene cause arRP [13].

Of the above results, the most important was the discovery in 1997 of the two new arRP genes—cellular retinaldehyde-binding protein (CRABP) and RPE65—both of which are expressed in the retinal epithelium (RPE) and both also involved in vitamin A metabolism. The two new arRP genes were reported in two back-to-back papers published in the leading journal in this field *Nature Genetics* in October 1997 [8, 9]. The discovery that these two genes were responsible for arRP provided the *first evidence that defects in the RPE cells and or in the vitamin A cycle may cause arRP.* Until this finding all the genes previously implicated in RP, since the early 1980s, had been genes expressed in the photoreceptor cell layer or the neuroretina. Because the RPE is a far simpler structure, the neuroretina defects in the RPE are more amenable to treatment (via vit A administration, drugs, transplantation, gene therapy, etc.). Consequently by opening up the possibility of the development of therapies for at least a proportion of patients with these diseases, this result transformed the outlook for sufferers with inherited retinal dystrophies. The finding was discussed in an editorial review in *Nature Genetics* issue of 17th October 1997 and reported in the *New Scientist* in the October 4 issue 1997. One very recent consequence was the report of the first successful use of gene therapy for a genetic eye disease, carried out at Moorfields Eye Hospital [2008], where patients received a healthy copy of the gene RPE65. This was a landmark scientific advance and was listed in *Discovery* magazine as the 24th most important scientific result of 2008[1].

The overall success of the project over the first decade is indicated by the fact that as a result of the study, a total of eight new disease genes had been located by 1999, including six new non-syndromal arRP genes. [In 1999 these six genes represented a significant fraction of all then known non-syndromal arRP loci. (Note that in March 1999 there were 16 known loci in which mutations cause non-syndromal arRP, the 14 arRP loci cited on the RetNet website, 11 March 1999, plus the two unpublished loci from the University of Otago mentioned above).]

After working with MJD and his team for a while at the Otago, GKM wanted to move back to India to run an ocular genetics laboratory and counselling unit. On returning to India, he was asked by Dr. Badrinath and Dr. HN Madhavan, the then research director, to set up a gene mapping/molecular genetics lab at Nethralaya, which he finally established with the support of MJD and Dr. Peter Swarbrick from Otago University,

[1]See Discover magazine published on line 17 Dec 2008. http://discovermagazine.com/columns/top-100-stories-of-2008/

New Zealand. GKM later went to join Fielding's laboratory at the National Eye Institute to learn the then modern fluorescent-based gene mapping and sequencing technology. Setting up the lab at Nethralaya was a great progress and addition to the institute. What proved a propitious move frutioned, as the lab went on to be perhaps the most productive in the area of ophthalmic genetics in India (with more than 50 publications) and one of the important ophthalmic genetic centres in the world, where thousands of patients were counselled and benefitted. What results emerged from the bedside to the bench finally reached the bedside again!

Starting as a small and precarious research project in 1990s, we went on to be become one of the most successful RP projects in the world. What started off in India finally came back to the country by the establishment of an ocular genetics laboratory and counselling unit, in the generous effort to fight against blindness and provide vision and light to the visually impaired patients.

Conflict of Interest None of the authors have any proprietary interests or conflicts of interest related to this submission.

Compliance with Ethical Requirements The whole journey and scientific endeavours were in complican with the ethical requirements.

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12 Panel-Based Next-Generation Sequencing for Inherited Retinal Degenerations in Koreans

Sang Jin Kim

Abstract

Inherited retinal degenerations (IRDs) include various forms of blinding retinal degeneration with genetic heterogeneity such as retinitis pigmentosa, cone-rod dystrophy, Stargardt disease, etc. Establishing a molecular diagnosis in IRD is important for proper diagnosis, genetic counseling, predicting prognosis, and clinical trials of retinal gene therapies. In this chapter, recent studies using gene panel-based next-generation sequencing in Korean patients with IRDs will be presented. Targeted gene panel sequencing seems to be an efficient approach to find genetic causes of IRDs.

Keywords

Choroideremia · Cone-rod dystrophy · Next-generation sequencing · Retinitis pigmentosa · Stargardt-like macular dystrophy · Targeted sequencing

12.1 Introduction

Inherited retinal degenerations (IRDs) are one of the most common forms of inherited human diseases worldwide. IRDs include various forms of retinal degeneration such as retinitis pigmentosa (RP), cone-rod dystrophy (CRD), Stargardt disease, choroideremia, etc. IRDs often cause serious visual impairment including legal blindness and severe constriction of visual field. Moreover, IRDs are associated with socioeconomic problems such as psychosocial difficulties, physical inactivity, depressive mood, and poor mental health [1, 2]. To prevent further retinal damage and to improve visual function, gene therapy has been pursued for a long period of time. Recently, many clinical trials of gene therapy have been performed for the treatment of IRDs [3, 4]. Most of these clinical trials have been performed in Western countries. One of the reasons for low number of clinical trials in Asian countries may be due to lack of extensive genetic studies to identify causal mutations in Asian countries. Promising results from clinical trials of retinal gene therapy for IRDs emphasizes the importance of genetic studies to find target genes in IRDs and eligible patients with IRDs. In this chapter, we described the panel-based next-generation sequencing in Korean patients with IRDs.

S. J. Kim (✉)
Department of Ophthalmology, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea
e-mail: sangjinkim@skku.edu

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12.2 Epidemiology of RP in Korea

RP is the most common form of IRDs. The worldwide prevalence of RP has been reported to be 1 in 3000–7000 [5]. Recently, nationwide population-based studies on epidemiology of retinitis pigmentosa in Koreans have been published. In a population-based study from 2011 to 2014 in Korea, the incidence rate of RP for all ages was found to be 1.64 cases per 100,000 person-years [6]. The incidence of RP distribution showed one smaller peak observed in the 20–24-year-old age group (1.24 cases/100,000 person-years) and a larger peak observed in the 65–69-year-old age group (3.26 cases/100,000 person-years) [6]. The overall incidence was similar in men and women (1.64 versus 1.63 cases/100,000 person-years for men and women, respectively) [6]. Another population-based retrospective cohort study from 2011 to 2014 found that the prevalence of RP in Korea is about 1 in 9000 for all ages and 1 in 6000 for those over 40 years of age [7]. The mean age at diagnosis was 44.8 years and was 6 years earlier for male than for female patients (41.9 versus 47.8 years) [7]. The standardized mortality ratio was 1.56 for all ages, peaking at 2.61 in men aged 40–59, which was mainly attributed to 6.6-fold higher suicide rates than the same age group in the general male population [7].

12.3 Panel-Based Next-Generation Sequencing (NGS) (1): Methods

Gene panel for IRDs usually consists of genes known to cause IRDs. The gene panel needs to be updated regularly to include newly identified genes associated with IRD. From 2013 to 2015, we used a gene panel of 98 genes, which includes *DHDDS*, *RPE65*, *ABCA4*, *PRPF3*, *SEMA4A*, *PDC*, *CRB1*, *NEK2*, *FLVCR1*, *USH2A*, *ZNF513*, *C2ORF71*, *EFEMP1*, *FAM161A*, *SNRNP200*, *CNNM4*, *MERTK*, *CERKL*, *SAG*, *ARL6*, *IMPG2*, *RHO*, *CLRN1*, *PDE6B*, *RAB28*, *PROM1*, *GPR125*, *WDR19*, *CNGA1*, *LRAT*, *PDE6A*, *MAK*, *TULP1*, *GUCA1A*, *GUCA1B*, *PRPH2*, *EYS*, *RIMS1*, *IMPG1*, *ELOVL4*, *KLHL7*, *RP9*, *IMPDH1*, *KIAA1549*, *RP1L1*, *ADAM9*, *RP1*, *CNGB3*, *C8ORF37*, *KCNV2*, *TOPORS*, *PRPF4*, *RBP3*, *CDHR1*, *RGR*, *PDE6C*, *BEST1*, *ROM1*, *MYO7A*, *C1QTNF5*, *CACNA2D4*, *PDE6H*, *RDH5*, *MVK*, *RPGRIP1*, *NRL*, *RDH12*, *SPATA7*, *TTC8*, *NR2E3*, *RLBP1*, *ARL2BP*, *CNGB1*, *PRPF8*, *AIPL1*, *PITPNM3*, *GUCY2D,UNC119*, *CA4*, *RGS9*, *USH1G*, *PRCD*, *FSCN2*, *PDE6G*, *RAX2*, *CRX*, *PRPF31*, *IDH3B*, *MKKS*, *PRPF6*, *C21ORF2*, *TIMP3*, *OFD1*, *RPGR*, *RP2*, *CACNA1F*, and *CHM* [8–10].

Targeted gene panel sequencing was performed as previously described [8–10]. Genomic DNA was obtained from peripheral blood of the patients and family members, if possible, after obtaining informed consent. Genomic DNAs within the target genes were captured using SureSelect customized kit (Agilent Technologies, Santa Clara, CA), and libraries were prepared using the SureSelect Target Enrichment System protocol. Targeted exome sequencing was performed on Illumina HiSeq 2500 platform (Illumina, San Diego, CA). Sequencing reads were aligned to the Genome Reference Consortium human genome (build37) using Burrows-Wheeler Aligner with MEM algorithm. Aligned SAM/BAM files were sorted and indexed by SAMTOOLS and duplicated reads were removed by Picard tools. Secondary reads alignment was performed following known insertions/deletions (indels) from gold standard (dbSNP138, Mills, 1000 Genome Project phase 1 indels) by Genome Analysis ToolKit (GATK). Base recalibration was performed and annotated by GATK. Single nucleotide variants (SNVs) and insertion or deletion (indels) were calculated by Unified Genotyper in GATK. ANNOVAR was used to annotate called variants from variant call format.

To identify candidate variants, following criteria were applied: (1) located in exonic region, (2) rare allele frequency (MAF <0.01 of The Exome Aggregation Consortium), (3) damaged in prediction tools (SIFT, Polyphen2, GERP++), and (4) clinical significance (ClinVar) [8–10].

Sanger sequencing was also performed to examine the presence of variants of interest in the proband and family members.

12.4 Panel-Based Next-Generation Sequencing (2): Application in Korean Patients with IRDs

12.4.1 Stargardt-Like Macular Dystrophy 4

Classical Stargardt disease (STGD1; OMIM# 248200) is caused by mutations of *ABCA4* gene on chromosome 1p with autosomal recessive transmission [11]. Families with autosomal dominant inheritance have also been described, including Stargardt-like macular dystrophy 3 (STGD3; OMIM# 600110) caused by mutations in *ELOVL4* and STGD4 (OMIM# 603786) caused by mutation in *PROM1* gene on chromosome 4 [12, 13].

STGD4 is characterized by bilateral bull's eye atrophy of macula and the presence of yellow flecks. STGD4 is caused by mutation in *PROM1* gene encoding prominin-1, a 5-transmembrane glycoprotein also known as CD133 [13]. It was suggested that prominin-1 was involved in photoreceptor disk morphogenesis [13]. *PROM1* c.1117C>T (p.R373C) missense mutation is known to cause several types of autosomal dominant retinal degeneration, including STGD4, bull's eye macular dystrophy (MCDR2), retinitis pigmentosa, and cone-rod dystrophy [13, 14].

We identified *PROM1* p.R373C mutation in a 38-year-old Korean man with a family history of visual impairment suggesting autosomal dominant inheritance (Fig. 12.1) [8]. Multimodal fundus imaging revealed bilateral atrophic macular lesions with small flecks in the posterior pole with a Bull's-eye pattern of hypo-autofluorescent macular lesions surrounded by hyper-autofluorescence [8]. Spectral-domain optical coherence tomography showed retinal pigment epithelium atrophy and photoreceptor layer defect (Fig. 12.1) [8]. Automated visual field test showed bilateral central scotoma [8]. Full-field electroretinogram (ERG) according to the International Society for Clinical Electrophysiology of Vision standards showed slightly reduced amplitudes in both cone and rod responses (Fig. 12.2) [8].

Targeted exome sequencing data were generated, covering 97.8% of targeted genes with a sequencing coverage of ≥ 100X as well as 99.3% of ≥ 50X. Targeted regions of *PROM1* were fully covered, with a mean depth ranging from 168X to 513X [8]. A heterozygous missense variant in *PROM1* (NM_006017.1: c.1117C>T (p.R373C)) was detected in the affected proband with a high read depth [8]. Sanger sequencing confirmed the heterozygous presence of the variant in the proband (Fig. 12.3) [8]. No suspicious pathogenic variant was identified from the other 97 genes [8]. From the characteristic retinal phenotype and *PROM1* p.R373C mutation, the patient was diagnosed as STGD4.

12.4.2 Choroideremia

Choroideremia is an X-linked disorder causing progressive degeneration of the retina, retinal pigment epithelium (RPE), and choroid [15]. Affected patients show night blindness with progressive peripheral vision loss and eventual central vision loss [16]. Female carriers may show patchy chorioretinal atrophy [17]. *CHM*, which encodes Rab Escort Protein-1 (REP-1), is a gene responsible for choroideremia. REP-1 facilitates posttranslational modification of Rab proteins regulating intracellular trafficking [18]. Various type of mutations in *CHM* have been identified including various size of deletions, nonsense mutations, missense mutations, frameshift mutations, splice site defects, and deletion of the entire gene, causing the truncation, loss of functional domain, or absence of REP-1 protein [19, 20].

Because choroideremia is frequently caused by large deletion of *CHM*, NGS alone may not make a proper molecular diagnosis for a considerable number of patients with choroideremia. Therefore, direct sequencing of *CHM* gene, RNA (cDNA) sequencing, or immunoblot to detect truncated or absent protein have been performed

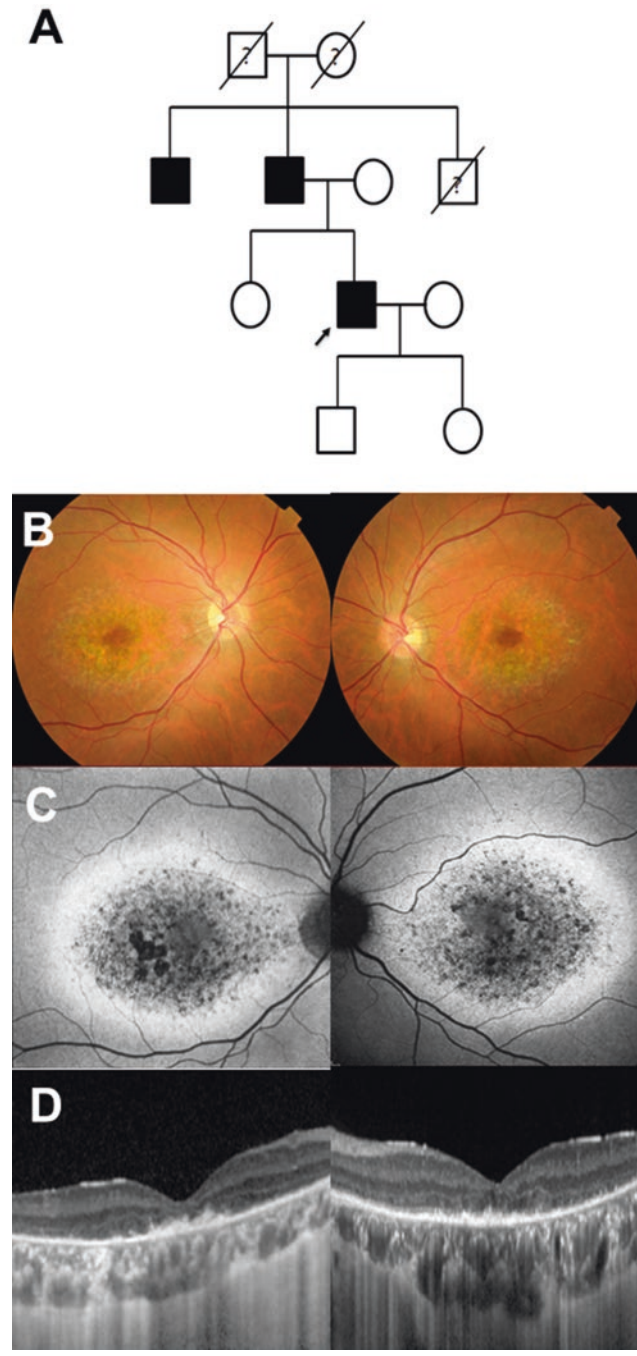

Fig. 12.1 Pedigree (**a**), fundus photographs (**b**), fundus autofluorescence photographs (**c**), and spectral-domain optical coherence tomography (**d**) of the affected proband with Stargardt-like macular dystrophy 4. (Reprinted from Ann Lab Med. 2017;37:536–539 with permission)

in cases with suspicious ocular phenotypes [20]. Recently, NGS-based approaches also have been reported with successful diagnosis of choroideremia [9, 21]. NGS-based approach may be particularly useful when it is difficult to clinically differentiate choroideremia from other inherited retinal degenerations.

Recently, *CHM* mutations were identified in two Korean families [9]. In one family, direct *CHM* sequencing was performed, and in the other

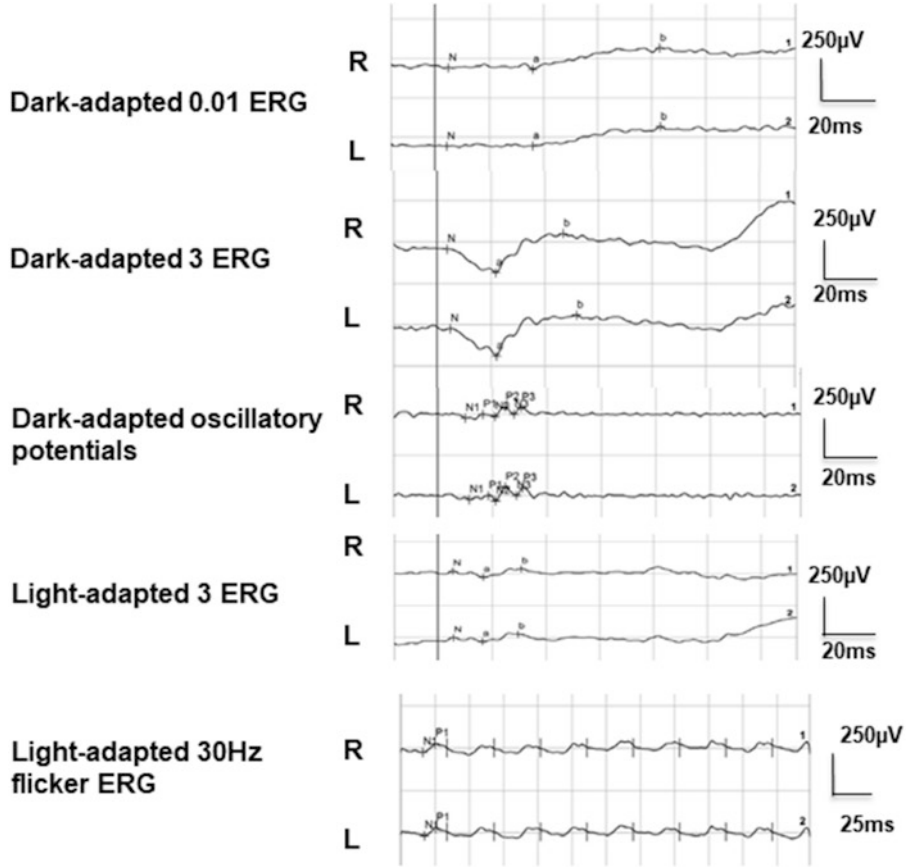

Fig. 12.2 Full-field standard electroretinogram in a Korean patient with Stargardt-like macular dystrophy 4. (Reprinted from Ann Lab Med. *2017;37:536–539* with permission)

family, panel-based NGS sequencing was done first, followed by direct *CHM* sequencing [9].

In family A, the proband was a 45-year-old man with night blindness, visual field defect, and decreased central vision [9]. The fundus exam showed bilateral chorioretinal atrophy and areas of RPE disruption with sparing of the central macula (Fig. 12.4) [9]. In fundus autofluorescence (FAF) photographs, residual RPE tissue appeared as a well-demarcated hyperfluorescent area (Fig. 12.4) [9]. Standard ERG showed almost extinguished cone and rod responses. SD-OCT showed retinal thinning, choriocapillary atrophy and abrupt transition to atrophic areas (Fig. 12.4) [9]. In family B, the proband was a 41-year-old man with night blindness and visual field defect. Findings of the fundus exam, FAF, and SD-OCT scans were similar to those of proband A (Fig. 12.4) [9].

In proband B, targeted sequencing of 98 candidate genes for retinal degenerations revealed no suspicious variations [9]. However, exon 9 of *CHM* was not captured at all, suggesting exon 9 deletion (Fig. 12.5) [9]. Direct *CHM* sequencing was also performed in the probands of family A and family B. Fifteen coding exons and their flanking introns were amplified by PCR, and the resultant amplicons were sequenced on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) [9].

In the proband of family A, 9 base pair deletion in exon 3 and adjacent intron sequences was identified (c.184_189 + 3delTACCAGGTA) (Fig. 12.6) [9]. In the proband of family B, PCR

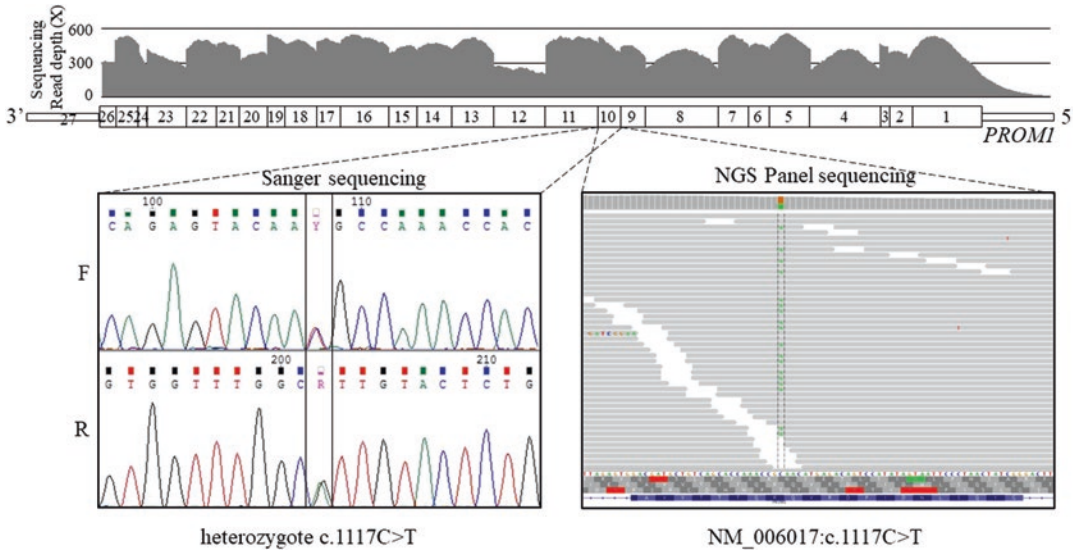

Fig. 12.3 DNA electropherograms of a fragment of *PROM1* gene by Sanger sequencing in a Korean patient with Stargardt-like macular dystrophy 4. Proband DNA sequence showing a heterozygous missense variant (NM_006017.1: c.1117C>T; p.R373C). (Reprinted from Ann Lab Med. 2017;37:536–539 with permission)

product of exon 9 was not detected, suggesting exon 9 deletion (Fig. 12.6) [9]. Sequencing analysis of other amplified products showed no pathogenic variant in the proband of family B [9].

12.4.3 Cone-Rod Dystrophy

CRD is a group of inherited retinal disorders presenting primary loss of cone photoreceptors and subsequent or simultaneous loss of rod photoreceptors. Patients with CRD show severely-impaired central visual acuity and color vision deficiency. Autosomal recessive, autosomal dominant, and X-linked forms of CRD have been reported. Among the variable genetic causes of autosomal recessive CRD, *ABCA4* mutations are the most common ranging from 24% to 65% [22–27].

Recently, a novel homozygous missense variant in exon 1 of *RAB28* was identified in a 11-year-old Korean patient with progressive visual impairment [10]. BCVA in both eyes was 20/100 [10]. The fundus examination showed slightly atrophic fovea, and SD-OCT showed an ellipsoid zone defect in both macular areas (Fig. 12.7) [10]. The Ishihara color vision test showed severe color vision deficiency [10]. Standard ERG revealed almost extinguished light-adapted 3 ERG and 30 Hz flicker ERG, and automated visual field test showed central scotoma in both eyes [10]. Thus, she was clinically diagnosed as having CRD.

Targeted exome sequencing data were generated with 2 million sequencing reads, covering 97.42% of 98 targeted genes with a sequencing coverage of ≥ 100X [10]. Targeted regions of *RAB28* were fully covered, with a mean depth ranging from 195X to 342X [10]. A novel homozygous missense variant (NM_004249.3:c.68C>T (p.Ser23Phe)) was detected in the proband, which was confirmed by Sanger sequencing [10]. Unaffected parents and an unaffected male sibling were heterozygous carriers of the missense variant [10]. In addition, no pathogenic variants were identified in the other 97 genes including 8 genes known to be associated with autosomal recessive CRD (*ABCA4*, *ADAM9*, *C8orf37*, *CERKL*, *EYS*, *PROM1*, *RPGRIP1*, and *TULP1*) [10].

This *RAB28* c.68C>T variant is thought to be a "likely pathogenic" variant in that (1) the variant is either absent or present at extremely low frequency in several population databases, [2]

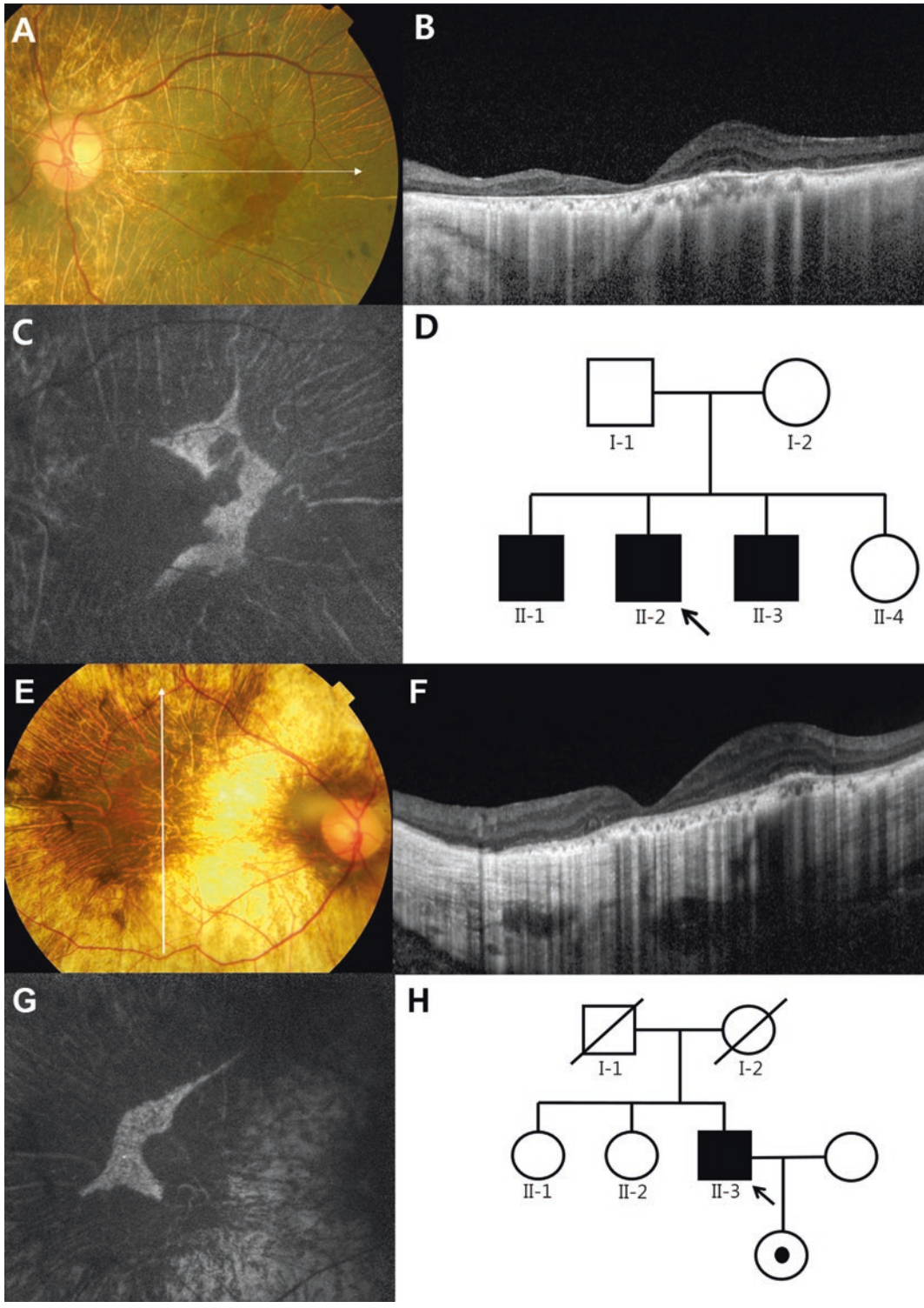

Fig. 12.4 Ocular phenotypes shown in the probands with choroideremia and the pedigrees of family A (**a**–**d**) and family B (**e**–**h**). (**a**, **e**) Fundus photograph. (**b**, **f**) Spectral-domain optical coherence tomography. (**c**, **g**) Fundus autofluorescence photograph. (**d**, **h**) Pedigrees of the two families. (Reprinted from Ann Lab Med. 2017;37:438–442 with permission)

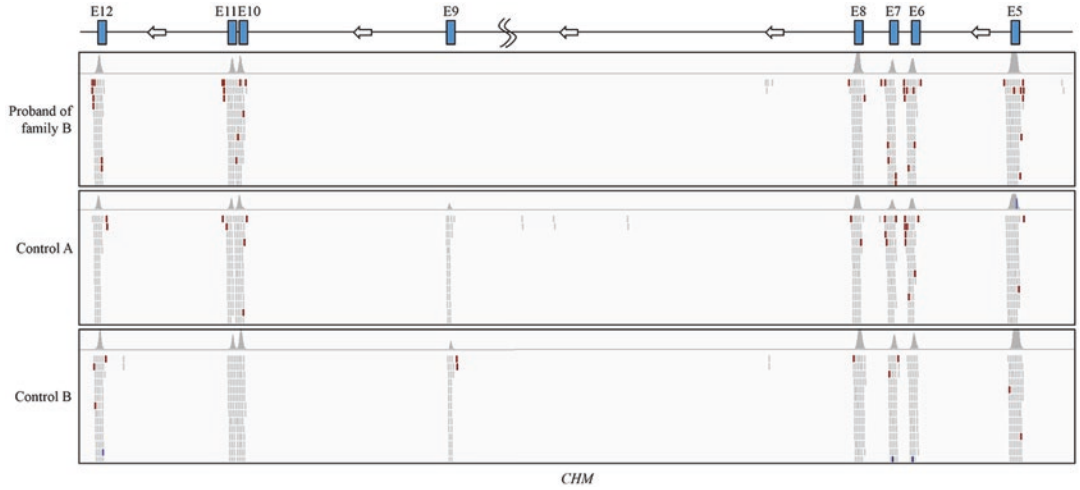

Fig. 12.5 BAM file created by using IGV viewer of targeted sequencing of 98 candidate genes including *CHM*. *CHM* exons were present in control subjects. On the contrary, *CHM* exon 9 is absent in proband B. (Reprinted from Ann Lab Med. 2017;37:438–442 with permission)

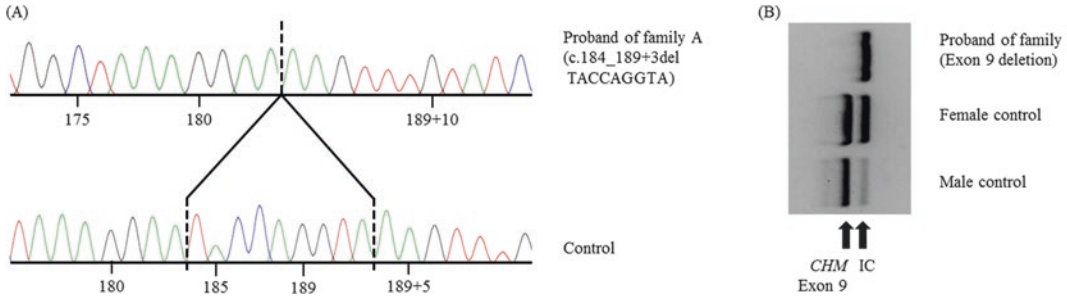

Fig. 12.6 *CHM* variants identified in the probands of family A and family B. (**a**) Chromatogram of c.184_189 + 3delTACCAGGTA (p.Tyr62_Gln63del) detected in the proband of family A. (**b**) PCR products of exon 9. Exon 9 deletion was detected in the proband of family B. (Reprinted from Ann Lab Med. 2017;37:438–442 with permission)

multiple prediction algorithms such as SIFT and PolyPhen-2 support a deleterious effect, [3] segregation of the *RAB28* mutation with the phenotype in the family was shown, [4] no pathogenic variants were identified in genes known to be associated with IRDs or AR-CRD, and [5] the variant was located in a well-established functional domain of the RAB28 protein [10].

RAB28 is a recently identified gene in autosomal recessive CRD [28–30]. *RAB28* encodes a member of the Rab subfamily of the RAS-related small GTPases [28]. Previously, only four *RAB28* mutations from four European families have been reported including nonsense mutations in exons 5 and 6, a splice donor site mutation in exon 2, and a missense mutation in exon 8 [29, 30].

12.5 Conclusion

In patients with IRDs, establishing a molecular diagnosis is important for proper diagnosis, genetic counseling, predicting prognosis, and clinical trials of retinal gene therapy. Because it is

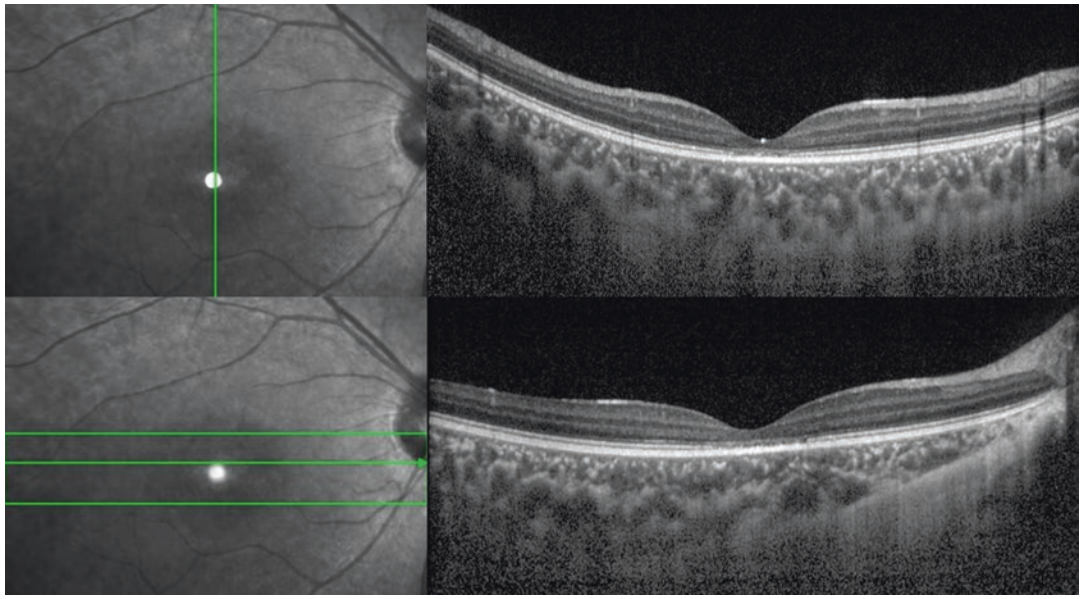

Fig. 12.7 Spectral domain optical coherence tomography images in a Korean cone-rod dystrophy patient with *RAB28* mutation

uncommonly possible to predict the causative gene, mass screening is inevitable. NGS has accelerated finding causative mutations in Mendelian diseases such as IRDs. Targeted gene panel sequencing seems to be an efficient approach to find genetic causes of IRDs. Further studies including verification in large-scale studies and comparison with whole exome or whole genome sequencing are warranted.

Compliance with Ethical Requirements Author Sang Jin Kim declares that he has no conflict of interest.All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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13 Genetic Disease in Ophthalmology: Healthcare and Research Opportunity in Bangladesh

A. H. M. Enayet Hussain and Khaleda Islam

Abstract

Genetic eye diseases which may pass on from parent to children through genes include a large number of ocular pathologies all of which do not cause visual impairment. Global as well as country burden of the problem is unknown. Consanguineous marriage is common in the country which is a predisposing factor for the genetic eye diseases. Bangladesh is striving to develop reliable infrastructure of eye healthcare delivery system from community clinic providing preventive and promotive care to tertiary level facilities and specialized institutes providing specific treatments. Utilizing the field staffs, eye patients may be screened at the community and referred to the health facilities for further diagnosis and treatment. Sociodemographic data may reveal the consanguineous marriage history and will help diagnosing genetic eye diseases. Thus proper utilization of health system will not only ensure eye care of patients but also to create opportunities of research with data generation providing magnitude of eye disease with a focus to genetic eye disorder in this large population.

Keywords

Ocular genetic disease · Autosomal recessive · Autosomal dominant · Health system · Primary healthcare · Consanguineous marriage

13.1 Introduction

Genetic eye diseases include a large number of ocular pathologies that have in common the transmission from parents to children by their genetic inheritance but all do not cause visual impairment [1]. A genetic or inherited disease may pass on from parent to their children through the coded information in the genes, which determines the type of genetic disorder. When involved genes are located on the chromosomes numbered 1 to 22, it is called autosomal, and when resided on the sex chromosomes, it is called X-linked or sex-linked. The characteristics of X-linked inheritance are that males are affected whereas females are usually unaffected carriers.

Genetic factors are responsible for over 60% of childhood blindness (congenital glaucoma, ocular malformations, atrophy of the optic nerve, retinitis pigmentosa, etc.), also associated with serious eye diseases, including glaucoma and macular degeneration in adults. Some common

A. H. M. Enayet Hussain (✉) · K. Islam
Directorate General of Health Services, Mohakhali, Dhaka, Bangladesh

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ocular genetic diseases that are found here are as follows [2]:

- Corneal dystrophies.
- Anterior segment dysgenesis.
- Aniridia.
- Uveal melanoma.
- Glaucoma.
- Cataract.
- Norrie disease.
- Retinoschisis.
- Retinitis pigmentosa.
- Choroideremia.
- Albinism.
- Color vision deficiencies.
- Inherited optic neuropathies.
- Anophthalmia.
- Keratoconus.
- Marfan syndrome.
- Stargardt disease.
- Pseudoexfoliation syndrome.

13.2 Epidemiology

Global burden of visual impairment from genetic causes is not known though it seems that in industrialized countries significant percentage of blindness is due to genetic cause [1]. No literature is found yet on genetic eye disease in Bangladesh, though blindness is a major public health problem in the country and majority of which is preventable. National blindness and low vision survey of Bangladesh (2003) revealed that prevalence of bilateral blindness was 1.52% with an estimated 650,000 blind adults aged 30 years and older in population. The reasons were unoperated cataract (73.3%), refractive error (18.8%), macular degeneration (1.8%), uncorrected aphakia (1.1%), etc. The study recommended national eye plan for effective eye care services [3].

13.3 Bangladesh Initiatives

Ispahani Islamia Eye Institute and Hospital was the first structured initiative to address the eye healthcare in the country in 1960. Later, Bangladesh National Council for Blindness (BNSB) was formed in 1978 to implement National Eye Care Plan. The government demonstrated its commitment to prevent blindness and ratified vision 2020 – the Right to Sight in 2003 [4]. The Ministry of Health and Family Welfare (MOH&FW) following the global eye health action plan 2014–2019 (2013) emphasized better integration of eye health into national health plans [5]. Vision 2020 committees were formed in all districts, which enhanced eye care services through GO, NGO, and private partnership [6]. Moreover, an intervention package which was piloted in 2016 demonstrated that existing health system may be utilized to detect children *with avoidable childhood blindness at community* and manage through referral at the higher health facilities having ophthalmic unit [7]. Though the health system is challenged to ensure ophthalmic personnel, equipment, in handling existing cataract and emerging eye problems like diabetic retinopathy, ocular trauma, retinopathy of prematurity, etc., MOH&FW need to know prevailing types of eye diseases with magnitude and identify the gaps in health system to ensure universal eye care.

13.4 Eye Care and Research Opportunity for Genetic Eye Disorder

Bangladesh has unique health system and infrastructure in public sector starting from community clinic delivering primary healthcare (PHC) mostly preventive and promotive up to tertiary level facilities and national institutes offering specialized treatments to the patients. Utilizing this health system, the country has made progress in public health with a remarkable reduction of under 5 mortality between 1993 (<5 MR 133) and 2014 (<5 MR 46) and achieved the Millennium Development Goal (MDG) 4 well ahead of time; the target was 48 deaths per 1000 live births by 2015.

The success story is indebted to the public health impact of several programs like expanded program of immunization (EPI) where all basic

vaccine coverage by age 12 months between 2004 and 2014 increased from 68% to 78%, nutrition program where children under age 5 showed reduction in stunting between 2004 and 2014 from 51% to 36%, maternal health program which decreased total fertility rate (TFR) from 6.3 (1975) to 2.3 (2014) children born per woman [8], and also reduced maternal mortality since 1990 to 2010 with an estimated reduction of 66% [9]. Utilizing the same health system eye care can be ensured along with identification and management of genetic eye disorder and creating research opportunity in the field.

Currently the primary level health infrastructure is the community clinic (CC), operated by Community Health Care Provider (CHCP), situated within half an hour walking distance of the people and mandated to serve 6000 catchment population. There are more than 13,000 operational CCs each of which is managed by Community Group and supported by 3 community support groups. In 4th Health, Population, Nutrition (HPN) sector program, five multipurpose community health volunteers (CHVs) are being recruited for each CC constituting a total number of 65,000 CHVs for the whole country. They will be working as bridge between community and health facility in addition to providing preventive and promotive eye care [6, 10, 11].

At the Upazila (subdistrict) health complex (UHC), there are field staffs attached to the CC, along with CHCP and multipurpose CHVs. They would be trained to screen eye patients while doing their routine domiciliary visits and referring patients to the UHC for diagnosis and management. The doctors, nurses, and paramedics of UHC would be trained on ophthalmic management, and the complicated cases would be referred to district or tertiary hospitals or at national level where there are National Institute of Ophthalmology and Hospital, Ispahani Islamia Eye Institute and Hospital (IIEI&H), specialized eye hospitals, and medical colleges. These national level institutes are from government, NGO, and private sectors having ophthalmologic units with ophthalmologists to confirm diagnosis and provide specific treatment and contributing substantially to the management of referred eye patients from all over the country.

Suspected case identification by frontline health workers will provide an idea about case load at community. Confirming diagnosis and treating the patient at ophthalmologic unit will provide case-based proportion of eye disease including genetic eye disorder attending the facility. Sociodemographic and family history will explore genetic eye disorder which is related to families and prevalent in offspring of consanguineous marriage. This is prevalent in the South Asian community in Great Britain and some other parts of world where they migrate. Consanguineous marriage is also common in Bangladesh, and the reason behind is strong cultural issues like cultural possessiveness, property inheritance, religion, and ethnicity.

Reddy et al. in their study identified that in East London in the 1980s, over 50% of marriages in the Pakistani community were consanguineous compared to 1% in general population and the children of consanguineous unions have increased postneonatal mortality and childhood morbidity compared with other ethnic groups. The predominant ethnic community in East London is Bangladeshi having tradition of consanguineous marriages. The study identified 13% of children (45/342) with an ocular genetic disorder or possible ocular genetic disorder; of them 22% (10/45) had a history of consanguinity with an inheritance pattern of 30% autosomal recessive (3/10), 20% autosomal dominant, and 50% X-linked/unknown/isolated cases. In the remaining non-consanguineous families (35/45), 22% were autosomal recessive, 17% autosomal dominant, and 60% X-linked/unknown/isolated cases. The vast majority of cases (9/10) with a history of consanguineous marriage had South Asian ancestry. The predominant inheritance pattern of ocular genetic disorders in patients with consanguineous parents is expected to be autosomal recessive [12].

Two-thirds of childhood blindness in the Middle East, with a prevalence ranging from 47% in Tunisia to 86% in Kuwait, is due to genetic diseases (autosomal recessive disorders), attributed to high rates of consanguineous marriage. Genetic eye disease is also common in Egypt (40%) and assumed to cause at least half of all cases of childhood blindness. Ahmed Gomaa assessed availability and use of genetic counseling services in Egypt, evaluated parents' attitudes and satisfaction with these services, and also assessed ethno-cultural beliefs about the causes of genetic disorders. He found that parents' perceptions of genetic disease varied; many attributed the condition to will of Allah, though majority understood it as a condition inherited within family. They were compliant with doctors' advice and wanted to discover risk of having another affected child, without understanding level of risk. Abortion is prohibited in Islam, and mothers were often blamed for their child's blindness with consequences like divorce, husband taking another wife, social stigma, having no more children, and financial difficulties. The study found that main barriers to service uptake were lack of motivation, cost, long waiting lists, distance, lack of awareness among doctors, and insufficient services [13]. While ensuring eye care, these types of studies can be conducted in Bangladesh through existing health system.

13.5 Summary

Proper utilization of existing health system will not only ensure screening of eye disorder at the community, early diagnosis, and timely treatment but will also provide information related to eye disorder with a focus on genetic causes. Genetic counseling service may be established for consanguineous marriage, which may help the parents to understand the risk of having children with genetic eye disease and deciding to have no more children. The program will not only help parents from getting rid of social stigma, psychosocial trauma, and financial difficulties but will also help the health system not to be overburdened with genetic eye disorders. Proper database will provide information regarding healthcare seeking pattern and success rate of treatment. Moreover sustainable strengthening of health system with mid-level eye personnel and basic eye equipment will also ensure universal health coverage of patients with eye disorder. However awareness creation of community, health literacy, strong community involvement, and community support are needed to maximize the efficiency of such programs.

Compliance with Ethical Requirements The authors A.H.M. Enayet Hussain and Khaleda Islam declares that they have no conflict of interest.

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Update on the Japan Eye Genetics Consortium (JEGC)

14

Takeshi Iwata

Abstract

Japan Eye Genetics Consortium (JEGC) was launched in 2011 to identify gene mutations responsible for 37 hereditary retinal diseases including hereditary optic neuropathy and hereditary glaucoma in Japanese population. More than 2,300 DNA samples have now been collected from 30 university ophthalmology departments in Japan for whole genome/exome analysis. Our study shows that approximately 80% of families with inherited retinal disease carry novel gene mutations. Number of new genes were identified and expected for dozens more. This high heterogynous genetic background of Japanese patients with novel gene mutations is a challenge to our consortium to identify all disease-causing mutations within the time frame of research funding. JEGC is also responsible to identify molecular mechanism of disease onset for each mutation and apply these seed information for therapeutic development.

Keywords

Japanese · Hereditary retinal disease · Optic neuropathy · Glaucoma · Gene mutation · Whole genome/exome sequence · Knock-in mouse · patient iPS cells

14.1 Launch of JEGC

JEGC was established by six ophthalmology departments, RIKEN (Yokohama), National Institute of Genetics (Mishima), and Tokyo Medical Center funded by the Japanese Ministry of Health, Labour and Welfare and later by the Japanese Agency for Medical Research and Development (AMED). In 2014, JEGC expanded to current size of 30 university ophthalmology departments when all board member of the Japanese Society for Clinical Electrophysiology of Vision (President, Prof. Masayuki Horiguchi, MD, PhD, Fujita Health University) joined the consortium (Table 14.1). Every 3 years the research grant has been renewed with additional objective for this consortium. The current research objective is to contenuously accumulate genotype-phenotype information of Japanese patients and to identify molecular mechanism for each mutant to develop potential therapeutic (Fig. 14.1).

T. Iwata (✉)
National Institute of Sensory Organs, Tokyo Medical Center, National Hospital Organization, Tokyo, Japan
e-mail: takeshi.iwata@kankakuki.go.jp;
http://iwatalab.org

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Table 14.1 List of JEGC member

Name	Institution
Takehi Iwata	National Hosipital Organization Tokyo Medical Center
Toshihide Nishimura	St. Marianna University
Yoshihide Hayashizaki	RIKEN (Institute of Physical and Chemical Research)
Kazusshige Tsunoda	National Hospital Organization Tokyo Medical Center
Mineo Kondo	Mie University
Miroko Tera saki	Nagoya University
Kei Shinoda	Saitama Medical University
Takaaki Hayashi	The Jikei University of Medicine
Kazuki Kuniyoshi	Kindai University
Shuhei Kameya	Nippon Medical School
Kaoru Fujinami	National Hosipital Organization Tokyo Medical Center
Nobuhiro Shimozawa	Tsukuba Primate Research Center, National Institute of Biomedical Innovation
Shinji Ueno	Nagoya University
Masayuki Hohguchi	Fujita Health University
SyuichiYamamoto	Chiba University
Manami Kuze	Matsusaka Central General Hospital
AtsushiMizota	Teikyo University
Nobuhisa Naoi	Miyazaki University
Shigeki MachkJa	Dokkyo Medical University, Koshigaya Hospital
YoshiakiShimada	Fujita Health University, Banbuntane Hotokukai Hospital
Makoto Nakamura	Kobe University
Hisashi Fujikado	Osaka University
Yoshihiro Hotta	Hamamatsu University School of Medicine
MasayoTakahashi	RIKEN (Institute of Physical and Chemical Research)
Kiyofumi Motiduki	Gifu University
Akira Murakami	Juntendo University
Hiroyuki Kondo	University of Occupational and Environmental Health
Susumu Ishida	Hokkaido University
Mitsuru Nakazawa	Hirosaki University
Teruhisa Hatase	Niigata University
Tatsuo Matsunaga	National Hospital Organization Tokyo Medical Center
Yozo Mryake	Aichi Mecical University
KazuoTsubota	Keio University
Akiko Maeda	Case Western Reserve University Department of Ophthalmology and Visual Sciences
Kosuke Noda	Hokkaido University
AtsuhiroTanigawa	Fujita Health University
Syuji Yamamoto	Jin Eye Clinic
Hiroyuki Yamamoto	Jin Eye Clinic
Makoto Araie	Kanto Central Hospital of the Mutual Aid Association of Public School Teachers
Makoto Aaihara	Tokyo University
Toru Nakazawa	Tohoku University
Tetsuju Sekiryu	Fukushima Medical University
Kenji Kashiwagi	University of Yamanashi
Kenjiro Kozaki	Keio University
Caminci Piero	RIKEN (Institute of Physical and Chemical Research)
Takeo Fukuchi	Niigata University
Atsuhshi Hayashi	University of Toyama
Katsuhiro Hosono	Hamamatsu University School of Medicine
Keisuke Mori	International University of Health and Welfare
Kazutoshi Yoshitake	Tokyo University
Yuriko Minegishi	National Hospital Organization Tokyo Medical Center
Daisuke lejima	National Hospital Organization Tokyo Medical Center
Aakiko Suga	National Hospital Organization Tokyo Medical Center
Brian P. Rossmiller	National Hospital Organization Tokyo Medical Center
Mao Nakayama	National Hospital Organization Tokyo Medical Center
Yu Teruyama	National Hospital Organization Tokyo Medical Center
Eiko Sanbe	National Hospital Organization Tokyo Medical Center
Natsuko Nakamura	Teikyo University
Go Mawatah	Miyazaki University
Kentaro Kurata	Hamamatsu University School of Medicine
Norihiro Yamada	Saitama Medical University

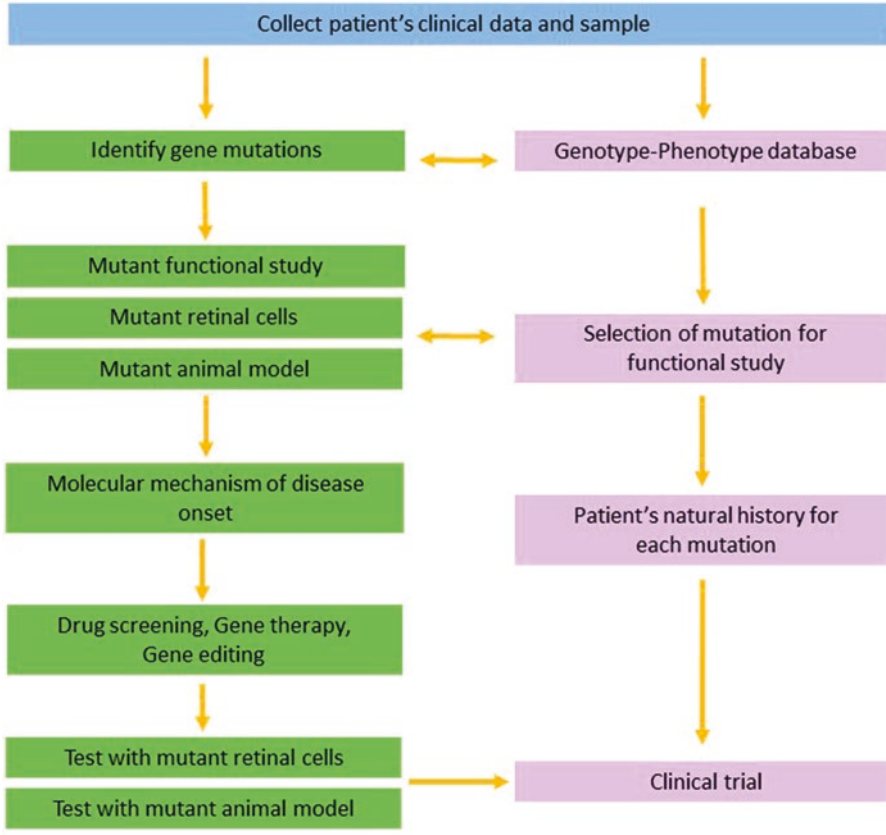

Fig. 14.1 Scheme of JEGC objective to collect family samples for clinical trials. Both functional study and collection of phenotypic information are time-consuming process requiring dedicated clinicians and scientists

14.2 JEGC Targeted Retinal Diseases

JEGC covers 37 retinal diseases (1. Leber congenital amaurosis, 2. retinitis pigmentosa, 3. enhanced S-cone syndrome, 4. Usher syndrome, 5. Stargardt disease (STGD), 6. macular dystrophy (non-STGD) or cone (cone-rod) dystrophy, 7. occult macular dystrophy, 8. cone (cone-rod) dystrophy with normal fundus appearance, 9. North Carolina macular dystrophy, 10. foveal hypoplasia, 11. microphthalmus/nanophthalmus, 12. congenital stationary night blindness (CSNB), 13. Oguchi's disease, 14. flecked retina syndrome, 15. Bietti crystalline corneoretinal dystrophy, 16. choroideremia, 17. achromatopsia, 18. blue cone monochromatism, 19. gyrate atrophy, 20. bradyopsia, 21. retinoschisis, 22. familial drusen, 23. familial AMD, 24. bestrophinopathy, 25. Wnt signaling retinopathy, 26. Stickler syndrome, 27. Wagner syndrome, 28. dominant optic atrophy (DOA), 29. mitochondrial retinopathy, 30. Leber's hereditary optic neuropathy (LHON), 31. ocular albinism, 32. oculocutaneous albinism, 33. albinism with systemic abnormalities, 34. angioid streaks, 35. retinoblastoma, 36. hereditary optic neuropathy, 37. hereditary glaucoma) for collection of genotypic and phenotypic information of patient and family (Fig. 14.2). Phenotypic characterization is examined by fundus photograph, optical coherence tomography, electroretinography, fluorescein angiography, autofluorescence, and other diagnostic methods.

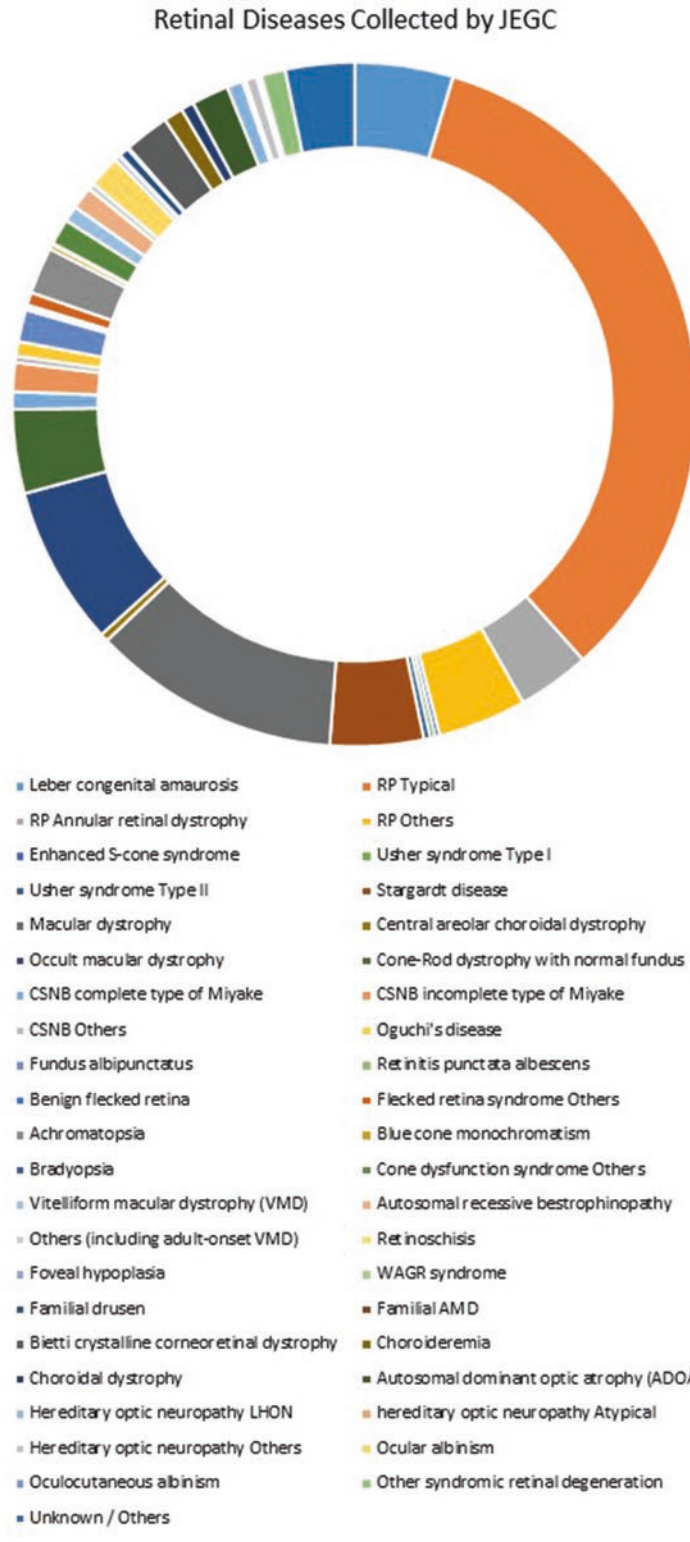

Fig. 14.2 Retinal diseases collected by JEGC. Over 1,300 pedigrees are currently collected aiming for 5,000, which is estimated to cover 10% of total Japanese patients

14.3 JEGC Diagnostic and Genotype-Phenotype Database for Deep Learning

All 37 eye diseases (Fig. 14.2) were selected by JEGC to collect information on patient's family phenotype information and blood/saliva samples for DNA extraction. The consortium came to an agreement that the phenotypic information is critical for grouping of patients with similar phenotype, for natural history study, and patient recruitment to clinical trials. To maintain high diagnostic quality, "disease leader" was appointed to ophthalmologist with experience for selected retinal disease. Disease leaders gather twice a year at Tokyo Medical Center to exchange information and to improve protocol for data collection. Online JEGC Genotype-Phenotype Database was modified in 2017 with replacement of Japanese menus to English for future use as Global Eye Genetics Consortium (GEGC) Genotype-Phenotype Database (Fig. 14.3). Currently, genotype-phenotype information of 2,300 individual are now entered into the system. The system is constantly reprogrammed for easier input and quicker output of genotype-phenotype data. In 2018, artificial intelligence (AI) server (IBM Japan, AI Vision) was connected to the database for deep learning of collected genotype and phenotype. The aim of this AI connection is to create a diagnostic support system for rare retinal disease and to improve genetic analysis to extract most likely disease-causing gene mutation from the whole genome/whole exome analysis. Linking AI drug discovery system is also in consideration. Higher quality of genotypic and phenotypic data is critical for the deep learning process.

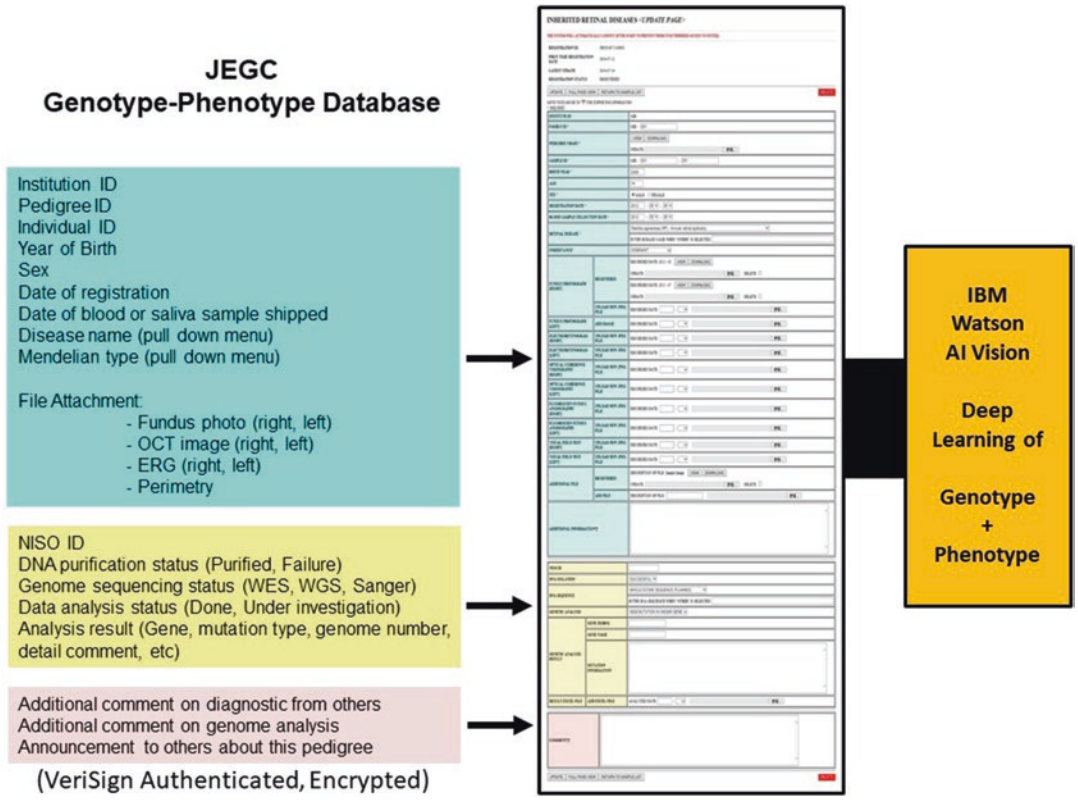

Fig. 14.3 JEGC Genotype-Phenotype Database was developed to collect data and to it transparent to JEGC members. The database is now linked with artificial intelligence server for deep learning of these rare retinal diseases

14.4 Identification of Novel Gene Mutations for Retinal Diseases

14.4.1 Identification of Novel Gene *CCT2* Responsible for Leber Congenital Amaurosis

Leber congenital amaurosis (LCA) is a hereditary early-onset retinal dystrophy with severe macular degeneration. In collaboration with Prof. Xunlun Sheng, Director of Ningxia Eye Hospital in China, we identified novel compound heterozygous mutations in *chaperonin-containing TCP-1, subunit 2 (CCT2)* gene, which encodes the chaperone protein CCTβ [1]. This CCTβ protein is a component of eight subunits (CCT α-θ) forming a two-ring structure of molecular chaperone. Previous study has shown zebrafish mutants of CCTβ exhibit abnormal eye phenotype, while its mutation and association with human disease have not been reported [2]. The identified novel CCTβ mutants T400P and R516H are biochemically instable, and affinity for adjacent subunit CCTγ was significantly affected in both mutants. The patient-derived induced pluripotent stem cells (iPSCs) carrying these CCTβ mutants were less proliferative than the control iPSCs. Decreased proliferation under *Cct2* knockdown in 661 W cells was significantly rescued by wild-type CCTβ expression. However, the expression of T400P and R516H didn't exhibit the significant effect. In mouse retina, both CCTβ and CCTγ are expressed in the retinal ganglion cells and connecting cilium of photoreceptor cells. The *Cct2* knockdown decreased its major client protein, transducing β1 (Gβ1). Knock-in zebrafish [3] and mouse is now being developed for further pathological study.

14.4.2 Identification of Novel Gene C21orf2 Responsible for Retinitis Pigmentosa and Cone-Rod Dystrophy

C21orf2 encodes a ciliary protein expressed through developing and mature stage of the retina localized to the connecting cilia between outer and inner photoreceptor [4]. Whole exome analysis was performed on 147 families with retinal degenerations, and novel mutations in *C21orf2* were found in Japanese patients with autosomal recessive retinitis pigmentosa (arRP) with skeletal defects and with autosomal recessive cone-rod dystrophy (arCRD) [5]. The patients in each family carried compound heterozygous mutations p.V111 M and p.Y107H or homozygous mutation p.Y107C, respectively. Bother mutations are localized in the leucine-rich repeat C-terminal domain (LRRC) required for protein stabilization. The effect of the mutations was examined by *in vitro* assays. *In vitro* expression of mutant *C21orf2* cDNA showed reduced protein levels and abnormal cytoplasmic localization compared to the wild type. Since *C21orf2* is required for ciliogenesis, the data suggested that reduced levels of functional C21orf2 can induce photoreceptor degradation through abnormal cilia formation, leading to arRP or arCRD.

14.4.3 Novel Gene Mutations in Known Genes

Our data shows approximately 20% of family collected with retinal disease carries known gene mutation and approximately 20% with novel mutations in known disease genes [6–13]. Phenotypical characteristic in Japanese population with different genetics background are being studied in these families.

14.5 Effective Therapeutic Development Using Patient iPS Cells and Knock-In Mouse Model

JEGC objective is to identify molecular mechanisms for each disease-causing gene mutation, which will serve as seeds information to develop new drugs or to alternatively use drugs currently available for diseases other than eye. A number of tools are now available to mimic patient's condition *in vitro* and *in vivo*. These techniques

include use of patient iPS cells, which can be transformed to retinal pigment epithelial (RPE) cells, retinal ganglion cells (RGC), photoreceptors, and other retinal cell types. In these differentiated cells, mutant protein localization and behavior can be directly observed and compared with cells derived from normal individual. In JEGC, iPS cells are generated from patients with novel mutation or unique phenotype and stored in biobank for use among consortium members for functional studies.

The CRISPR/Cas9 system to generate knock-in animal has become important technique to confirm abnormal behavior of the mutant protein *in vivo*. This technique is currently applied to zebrafish, mice, and cynomolgus macaque monkeys to develop knock-in models. This technique is effective especially when amino acid sequence of mutant protein is highly homologous between human and targeted animal. Knock-in mouse can be generated in a month and begin observation for abnormal phenotype within a year. A number of knock-in mice for novel genes are being developed in JEGC and periodically examined by fundus photo, optical coherence tomography (OCT), and electroretinogram (ERG) for any abnormal change in retinal structure and function.

Optineurin (OPTN), a gene responsible for hereditary normal tension glaucoma (NTG), is well studied with myocilin, another gene responsible for hereditary glaucoma with high intraocular pressure (IOP) [14]. A Japanese family with OPTN E50K mutation was identified by Dr. Kazuhide Kawase at Gifu University, and patient iPS cells were generated to differentiate neural cells. We soon noticed that mutant OPTN protein was accumulating in the endoplasmic reticulum of the patient-derived neural cells [15]. Immunoprecipitation experiment of mutant and normal OPTN was performed independently to identify interacting proteins by proteomic analysis. This analysis resulted with mutant protein interaction with TANK-binding kinase 1 (TBK1). The inhibitor for TBK1, amlexanox, a FDA- and PMDA-approved drug for allergic rhinitis and asthma, was later used to successfully disassociate mutant OPTN-TBK1 interaction [16]. To test if this mutant OPTN-TBK1 disassociation can lead to improvement of the disease, OPTN E50K knock-in mice was generated by CRISPR/Cas9 [3]. We administered amlexanox by oral administration 5 days a week for 1 year, which showed significant neural protection compared to non-treated mice (Fig. 14.4). Amlexanox is currently prepared for clinical trial funded by the National Hospital Organization of Japan for NTG patients with OPTN mutations.

We are working on another successful transition of basic to translational research on another gene mutation in JEGC [17].

14.6 Discussion and Future Prospects

JEGC has now added proteomic and transcriptomic analysis to the genome analysis in 2017 to study molecular mechanism of each disease-causing mutation. The Genotype-Phenotype Database plays major role to accumulate basic research and clinical information in one place, and to use the same database with patient's natural history for patient recruitment for future clinical trials. Artificial intelligence (AI) server is now connected to this database for deep learning of genotype-phenotype correlation. We hope this process will help improve clinical diagnostic and improve identification of disease-causing gene mutation for selection of future treatments.

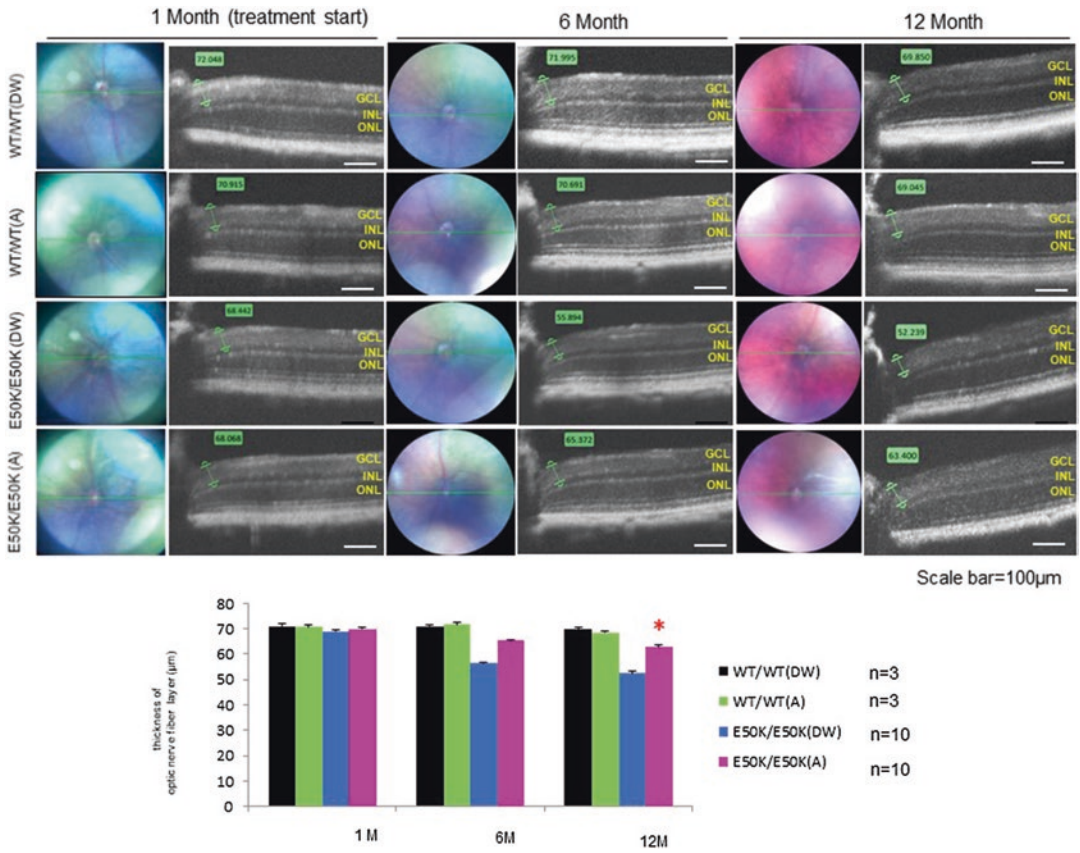

Fig. 14.4 Fundus and OCT image of OPTN E50K mutant and control treated or non-treated with amlexanox at 1, 6, and 12 months of age. Neuroprotection of amlexanox-treated NTG mice at 12 months (red asterisk). *DW:* distilled water, *A:* amlexanox

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Conflict of Interest Author of this chapter Takeshi Iwata has no conflict of interest.

Informed Consent All procedures in this research were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

Animal Study All institutional and national guidelines for the care and use of laboratory animals were followed.

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Genetics and Susceptibility of Retinal Eye Diseases in India

15

Sunita Mohan, Uthra Satagopan, Soumittra Nagasamy, Sundaram Natarajan, and Govindasamy Kumaramanickavel

Abstract

As per the World Health Organization, genetic eye disorders are one of the top ten major causes of global ocular health burden. AMD and DR take major share of the adult eye diseases component that particularly affects the neurovascular retina. More than 100 genes are known to cause Mendelian types of retinal degenerations including syndromic and non-syndromic RP, and it is presumed that this constitutes only 60% of all the genes known so far, and the remaining are yet to be identified. The burden of genetic disorders in India is significant, and very many significant genes like RPE65 have been identified with consanguineous autosomal recessive pedigrees obtained from this region.

Keywords

Leber congenital amaurosis · Phototransduction pathway · Retinitis pigmentosa · Stargardt disease · Visual cycle

S. Mohan (✉) · S. Natarajan · G. Kumaramanickavel
Aditya Jyot Foundation for Twinkling Little Eyes, Mumbai, India
e-mail: dr.sunitamohan282@gmail.com

U. Satagopan
Center for Medical Genetics, Chennai, India

S. Nagasamy
MedGenome, Bangalore, India

15.1 Introduction

Genetic eye disorders are one of the top ten causes of global ocular health burden (www.who.int/). It is increasingly evident that ophthalmic clinical practice by the physician would be deficient, if dealt without genetic knowledge and information [148]. Retinal diseases are one of the leading causes of childhood blindness [44], and inherited retinal disease has a prevalence of 1 in 4000 that is one of the common causes of blindness in children and working adults. Mendelian diseases such as retinitis pigmentosa (RP), congenital stationary night blindness (CSNB), and Stargardt disease have been reported to be caused by pathogenic genetic mutations, while adult onset retinal diseases such as age-related macular degeneration (AMD) and diabetic retinopathy (DR) have been shown to be hereditary through family-based studies and have been associated with variations in genes involved in the diseases' pathways. This review would provide overall information on the clinical and genetic aspects of retinal eye diseases, focusing on status of research in India in the field.

AMD and DR take major share of the adult eye diseases component that particularly affects the neurovascular retina. Over 19 genes/genomic regions cause susceptibility to AMD, out of which 2 genes complement factor H (*CFH*) and age-related maculopathy susceptibility 2 (*ARMS2*) have been widely reported, whereas the

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rest are minor common low effect alleles. However, environment factors like aging, diet, smoking, and hypertension contribute significantly to the disease onset and morbidity [55]. A number of single-locus genetic association studies and genome-wide association studies (GWAS) have helped in identifying few genes that may have a role in DR pathogenesis, yet the multifactorial involvement in DR pathology makes gene mapping quite challenging [74].

About 124 genes are known to cause Mendelian retinal degenerations including syndromic and non-syndromic RP, and it is presumed that this constitutes only 60% of all the genes known so far, and the remaining are yet to be identified [4, 37]. About 17 genes have been reported to be responsible for congenital stationary night blindness (CSNB) out of which some are unique to the disease, whereas some are common to RP as well, affecting both phototransduction pathway and visual cycle [163]. Vision sciences particularly genetics attracted intense studies, and hence so far 4000 mutations have been identified in the 124 genes (Table 15.1); genes like *ABCA4*, *BEST1*, *CDH23*, *CEP290*, *CRB1*, *EYS*, *MYO7A*, *OFD1*, *RPE65*, *RPGR*, *RHO*, *PRPH2*, *and USH2A* each have 100 mutations described with *ABCA4* alone having 700 mutations [27]. There is a high degree of clinical and genetic heterogeneity with overlapping phenotypes in retinal degeneration leaving a huge challenge in diagnostics, genetic counseling, and gene therapies [103]. Syndromic RP alone has 40 genes which are ciliopathies – Alstrom syndrome, Bardet-Biedl syndrome, Jeune syndrome or asphyxiating thoracic dystrophy, Joubert syndrome, and Meckel-Gruber syndrome. Zellweger is a spectrum of peroxisomal disorders caused by a dozen genes including Refsum disease. Kearns-Sayre syndrome and neuropathy-ataxia-RP (NARP) are caused by mitochondrial mutations [153]. The National Eye Institute has created a gene screening center and repository for patients with inherited ocular genetic disorders to facilitate and network scientists and clinicians to further research [45].

15.2 Epidemiology and Inheritance

Global prevalence of AMD is 8% (45–85 years) with a rising estimation of 196 million in 2020 to 288 million by 2040 and with a higher prevalence among Europeans compared to Asians [150]. DR affects 93 million people globally and is also expected to rise to alarming proportions in the next few decades [157]. In India the DR prevalence is around 15–18% among the diabetic population over 40 years of age [114, 139].

RP affects 1 in 3000–7000 people in a conservative estimate [18]. The prevalence in North China was found to be thrice higher when compared to rural Beijing (≥40 years [57, 159]). However in India it appears to be more frequent. A prevalence of about 1 in 750 in rural central India (≥30 years, [101]), 1 in 930 in urban South India, and 1 in 372 in rural India has been estimated for RP (≥40 years, [127]); the reason for such high prevalence is not clear but could be due to consanguineous marital practice that is widely prevalent in southern India. Consanguinity adds to the proportion of autosomal recessive eye diseases in the Indian population [73, 104]. Consanguineous marriages not only in South Asia and North Africa but also in London, due to the South Asian and North African immigrant populations, are causing different counseling approaches in pediatric clinic for patients with ocular genetic disorders [116].

15.3 Phototransduction Pathway and Visual Cycle

Electromagnetic photo-energy is bioprocessed to a neuroelectrical impulse in the retina to be perceived in the occipital lobe as vision. The physiology and biochemistry of vision are broadly divided into phototransduction (PT) in the neuroretina and visual cycle (VC) in the retinal pigment epithelium (RPE). In PT the final outcome is a neuroelectrical impulse, whereas in VC it is

Table 15.1 The list of retinal degenerative diseases genes classified based on the physiology and biochemistry of the neuroretina and retinal pigment epithelium, including syndromes

No	Function	Genes
1	Phototransduction cascade	***RHO*** (rhodopsin (G-protein coupled photon receptor)), ***PDE6A*** (rod cGMP-phosphodiesterase α-subunit (G-protein effector enzyme)), ***PDE6B*** (rod cGMP-phosphodiesterase β-subunit (G-protein effector enzyme)), ***CNGA1*** (rod cGMP-gated cation channel α-subunit), ***CNGB1*** (rod cGMP-gated cation channel β-subunit), ***SAG*** (arrestin (rhodopsin deactivation)), **GUCA1B** (guanylate cyclase activator 1B), **RDH12** (retinol dehydrogenase 12), **PDE6G** (phosphodiesterase 6G, CGMP-specific, rod, gamma)
2	Vitamin A metabolism	***ABCA4*** (ATP-binding cassette protein A4 [photoreceptor disc membrane flippase for vitamin A]), ***RLBP1*** (retinaldehyde-binding protein [11-cis-retinaldehyde carrier]), ***RPE65*** (retinal pigment epithelium 65, vitamin A trans-cis isomerase), ***LRAT*** (lecithin retinol acetyltransferase (synthesizes vitamin A esters)), ***RGR*** (RPE-vitamin A G-protein coupled receptor (photon receptor in RPE), **RBP3** (retinol-binding protein 3, interstitial), **MVK** (mevalonate kinase)
3	Structural or cytoskeletal elements	***RDS*** (peripherin (outer disc segment membrane protein)), ***ROM1*** (rod outer segment protein), ***FSCN2*** (fascin [actin-bundling protein]), ***TULP1*** (tubby-like protein), ***CRB1*** (crumbs homologue (transmembrane protein, adherent junctions)), **RP1** (microtubule-associated protein [microtubule formation and stabilization])
4	Signaling, cell-cell interaction, synaptic interaction, tissue development and maintenance	**NRL** (neural retina leucine zipper), **FAM161A** (family with sequence similarity 161, member A), ***SEMA4A*** (semaphorin B, transmembrane immune system protein), ***CDH23*** (cadherin 23 (adhesion receptor)), ***PCDH15*** (protocadherin 15 [adhesion receptor]), ***USH1C*** (Usher syndrome type IC ([integrating scaffold protein harmonin])), ***USH2A*** (Usher syndrome type IIA [Usher network protein]), ***MASS1*** (monogenic audiogenic seizure susceptibility 1 [Usher network protein]), ***USH3A*** (Usher syndrome type IIIA [transmembrane protein clarin 1]), ***RP2***, plasma membrane-associated protein
5	RNA intron-splicing factors	***PRPF31*** (precursor mRNA-processing factor 31 [spliceosome component]), ***PRPF8*** (precursor mRNA-processing factor 8 [spliceosome component]), ***PRPF3*** (precursor mRNA-processing factor 3 [spliceosome component]), ***RP9*** (PIM1-associated protein [RNA splicing factor]), **PRPF4** (pre-MRNA processing factor 4), **PRPF6** (pre-MRNA processing factor 6), **SNRNP200** (Small Nuclear Ribonucleoprotein 200kDa (U5)), **DHX38** (DEAH (Asp-Glu-Ala-His) box polypeptide 38))
6	Trafficking of intracellular proteins	**MYO7A** (myosin 7A [melanosome motility protein]), ***USH1G*** scaffold protein-containing ankyrin repeats and SAM domain [Usher's type I protein traffic regulator]
7	Maintenance of cilia/ ciliated cells (possible role in intracellular trafficking)	***BBS1***, ***BBS2***, ***BBS4***, ***BBS5***, ***BBS7*** (Bardet-Biedl syndrome), ***ARL6*** (ADP-ribosylation factor-like), ***MKKS***, McKusick-Kaufman syndrome (BBS6 protein), ***TTC8*** (tetratricopeptide repeat domain 8), ***PTHB1 (parathyroid hormone-responsive B1), RPGR*** (ciliary protein, cargo of PDE6D for ciliary trafficking) (viii) pH regulation (choriocapillaris) – ***CA4*** (carbonic anhydrase IV (carbon dioxide/bicarbonate balance)
8	Phagocytosis	**MERTK**, mer tyrosine kinase proto-oncogene [RPE receptor involved in outer segment phagocytosis]
9	Transcription factors	**NR2E3** (nuclear receptor subfamily 2, group E, member 3), **CRX** (cone-rod homeobox), **ZNF513** (zinc finger protein 513)
10	Photoreceptor maintenance and function	**ARL2BP** (ADP-ribosylation factor-like 2 binding protein)
11	Regulator of cell growth	**IMPDH1** (inosine-5'-monophosphate dehydrogenase type I [guanine nucleotide synthesis])
12	Cellular structure	**IMPG2** (interphotoreceptor matrix proteoglycan 2), **MAK** (male germ cell-associated kinase), **PROM1** (prominin-like protein 10)

(continued)

Table 15.1 (continued)

No	Function	Genes
13	Photoreceptor structure	**CLRN1** (clarin 1), **DHDDS** (dehydrodolichyl diphosphate synthase)
14	Anion channel	**BEST1** (bestrophin 1)
15	Ubiquitin related	**KLHL7** (ubiquitin-proteasome protein degradation), **TOPORS** (ubiquitin-protein ligase)
16	Cell signaling	**EYS** (eyes shut homologue (*Drosophila*)), **CERKL** (ceramide kinase-like (ceramide converting enzyme))
17	Cell division	**NEK2** (NIMA (never in mitosis gene A)-related kinase 2)
18	Others	***BBS10*** (vertebrate-specific chaperonin-like protein), **C2orf71** (chromosome 2 open reading frame 71), **C8orf37** (chromosome 8 open reading frame 37), **PRCD** (progressive rod-cone degeneration), **SPATA7** (spermatogenesis associated 7), **EMC1** (ER membrane protein complex subunit 1), **GPR125** (G-protein coupled receptor 25), **KIAA1549** (UPF0606 family, protein coding gene), **SLC7A14** (solute carrier family 7, member 14)

Modified as per Refs. [39, 51, 103]

the recycling of vitamin A. Several genes function critically in both the components to effectively optimize both PT and VC, to perceive vision. The electrical impulse is generated in the photoreceptors (rods and cones) and transmitted through neurotransmitters to the bipolar cells and then to the occipital lobe through the visual pathway.

In dark, the photoreceptors are in a constant state of depolarization, and glutamate neurosignals pass from them to the bipolar cells. At this state the PRs have their calcium-gated channels open; hence, ions like calcium and sodium pass into them freely, whereas irrespective of this mechanism potassium constantly keeps passing out of them freely. However in light, these channels close and prevent Ca^{++}/Na^{++} from entering the PR, but the K^{++} continues to be expelled; this causes hyperpolarization of PR and initiates to release the neurotransmitters to send signal to the bipolar cells. PT is triggered by photon-striking retinal in the rhodopsin molecule to convert to the activated form, which allows the binding of transducin, to rhodopsin. α-Subunit of transducin is released from its β- and γ-subunits of transducin which then binds to phosphodiesterase; this step converts cyclic GMP to GMP and makes the opened cGMP-gated channels to close causing hyperpolarization.

15.4 Genetics Overview

The burden of genetic disorders in India is significant [111, 142]. Considerable amount of eye genetic studies has been performed in the Middle East [63]. Countries like Egypt are providing eye genetic counseling services [46]. Twenty-nine different mutations causing various forms of retinal degenerative disorders, with similar human clinical phenotypes, are found in canines starting from stationary night blindness to cone-rod degenerations, which are ideal to be used as models for preclinical trials [22].

15.4.1 Retinitis Pigmentosa

RP starts majority of times with night blindness, as an early symptom, and later leads on to legal or total blindness. The fundus image is classical with mid-periphery pepper-salt appearance pigmentation and attenuated arteries. The pigmentation appears due to the tilting of balance of outer segment disc shedding and RPE scavenging; in RP the shedding is overwhelming, leaving them to be seen as pigments in the fundus.

RP is inherited in all Mendelian forms, including syndromic and mitochondrial inheritances. Autosomal dominant (adRP – with a global aver-

age proportion of 30–40%) is more common in North America, autosomal recessive (arRP, 50–60%) in southern India, and X-linked recessive (xlRP, 5–15%) in Britain. aR and xl onset is quite early compared to aD, since the normal gene compensates for some time. RP genes have been classified in multiple ways, since there are 124 causative genes; however, the most simple way to classify is according to the physiology and biochemistry, like genes pertaining to phototransduction cascade, vitamin A metabolism, structural and cytoskeletal elements, signaling, RNA intron-splicing factors, trafficking of intracellular proteins, maintenance of cilia, phagocytosis, transcription factors, photoreceptor maintenance and function, regulator of cell growth, cellular structure, photoreceptor structure, anion channel, ubiquitin related, cell signaling, cell division, and others (Table 15.1. Modified as per [51, 103]).

15.4.1.1 RP Genetic Studies in India

There are few reports on autosomal recessive and autosomal dominant non-syndromic RP from India. Homozygosity mapping has been extensively employed to map either known or novel candidate loci/genes in autosomal recessive diseases in both consanguineous and non-consanguineous families. The first report from India on homozygosity mapping on arRP identified RP22 locus in two families [38]. However, the causative gene in this locus is yet to be identified. A second new locus, RP28 on chromosome 2p11–2p15, was identified in an Indian family with multiple consanguineous marriages again using homozygosity mapping [49]. The linkage to this 16cM region was further fine mapped to a minimum critical region of 1.06cM between the D2S2225 and D2S296 markers in a second Indian family [69]. *FAM161A* was identified as the candidate gene in the RP28 locus in 2010, using ChIP and parallel sequencing [76]. Further mutations in *FAM161A* were reported in two more consanguineous arRP families and one out of hundred sporadic Indian RP patients using whole exome sequencing [166]. Also, few earlier reports on homozygosity mapping using either microsatellite markers flanking the known candidate genes region or genome-wide SNP genotyping arrays (Illumina Infinium arrays) on consanguineous and non-consanguineous families have reiterated the efficiency of identifying the disease causative gene/variation in autosomal recessive disease using this methodology. These studies reported identification of homozygous regions common between the affected sibs in 5/10 families; homozygosity in 10/34 families followed by detection of mutations in TULP1, RLBP1, ABCA4, RPE65, and RP1 in 5 of these 10 families; and mutations in TULP1, NR2E3, MRFP, and SPATA7 in 4/26 arRP families, respectively [58–60, 75, 129].

Homozygosity mapping followed by whole exome sequencing (WES) or WES alone has been employed, and the causative genes were identified as *TTC8* and *CRB1* in each one and *EYS* in two arRP families, respectively. Analyzing 100 sporadic RP cases by WES has identified mutations in *CRB1* and *EYS* in 2 and 8 cases, respectively [32, 48, 158]. *RHO* gene, though is a candidate for ADRP, a homozygous missense mutation, has been reported in the same in an Indian arRP family [72]. Mutations in codon 345 (V>M/L) and 347 (P>S/A/R/Q/L/T) in *RHO* gene are most frequent cause of ADRP, as reported from studies on various ethnicities worldwide. However, screening 100 Indian RP patients from 76 families revealed V345M mutation in an AD family with 3 affected and in 1 sporadic case [33]. A complete gene screening of ADRP genes, *RHO*, *PRPF31*, *RP1*, and *IMPDH1* in 48 isolated and 53 ADRP families, revealed a missense mutation (p.Gly106Arg) and a deletion c.358_359delAA leading to a frameshift and predicted protein truncation p.(Lys120GlufsX122) in *RHO* gene in each one of an isolated case and a splice-site mutation IVS6+1G>A in *PRPF31* gene in an ADRP family. The splice mutation observed in the ADRP family presented with incomplete penetrance as one of the clinically normal individual too harbored the variation [43]. Similarly, in a report by Saini et al., a deletion mutation c.59_65del7 (p.Gly20AlafsX43) in *PRPF31* was seen in a large ADRP family with ten affected members and one unaffected consistent with high incomplete penetrance of the gene.

This deletion was absent in other unaffected members and ethnic-matched controls [122]. Linkage mapping in an ADRP family of 34 members with 14 affected mapped a novel locus on 6q23 with a disease co-segregating region of about 25 Mb and a maximum two-point LOD score of 3.8 [58–60]. There are no reports yet on xlRP from India.

15.4.2 Stargardt Disease

Stargardt disease (STGD) is an autosomal recessive form of juvenile hereditary macular degeneration characterized by bilateral discrete yellowish round or pisiform flecks around the macula at the level of RPE [54]. It is one of the commonly hereditary forms of juvenile macular degeneration with an estimated prevalence of 1 in 8000 to 10,000 individuals. In more than 95% of cases, STGD is caused by mutations in *ABCA4* gene, and the remaining 5% is caused by mutations in the *ELOVL4*, *PRPH2*, *BEST1*, or *PROM1* gene. *ABCA4* encodes a transmembrane transporter protein that is responsible for clearance of a retinoid intermediate of the visual cycle from the intradiscal lumen of the outer segments of the rods and the cones. The three-step pathophysiology in a mutated *ABCA4* gene includes a defective rim protein, encoded by mutated *ABCA4* gene causing an accumulation of toxic by-product A2E in the RPE cells leading to the death of photoreceptors [132, 165].

Fundus flavimaculatus (FFM) is an allelic subtype of Stargardt disease that has been associated with mutation in the *ABCA4* gene and the *PRPH2* gene. FFM has a later age of onset. If loss of visual acuity begins in the first two decades, the designation STGD is preferred; if it begins later in life and has a more progressive course, the term FFM is preferred [147]. No racial predilection has been observed. Men and women are equally affected. Both eyes are usually equally and symmetrically affected. STGD patients typically complain of decreased visual acuity, gradually diminishing to 20/200. Clinical presentation in STGD varies greatly in the age of onset, presenting symptoms and the fundus appearance. The variations in the clinical presentation are due to the interplay of three prime factors that vary among the patients: (i) severity of their *ABCA4* genotype, (ii) relative sensitivity of the foveal cones to the genotype, and (iii) the relative sensitivity of the RPE to the genotype [125].

Early manifestation may only consist of some yellowish flecks and a macula with a snail's slime aspect. In the later stages, the macula may show bull's eye pattern with RPE atrophy or a beaten-bronze atrophy aspect. The functional changes remain usually restricted to the posterior pole of the eye, but they sometimes also affect the peripheral retina. Fluorescein angiography plays a key role in the diagnosis of Stargardt's, as it evidences dark choroid (silence choroidien), a characteristic sign of the disease that probably results from the accumulation of lipofuscin in the RPE [5]. Also relative sparing of the peripapillary RPE is a fairly reliable diagnostic sign of *ABCA4*-associated retinal disease more evident of fluorescein angiography.

15.4.2.1 Stargardt Disease Genetic Studies in India

The only report of *ABCA4* and Stargardt disease from India is on screening 5 STGD patients using targeted NGS with 184 retinal genes panel. Mutations were identified in all five: four compound heterozygous and one homozygous that includes two novel mutations [9].

15.4.3 Vitelliform Macular Dystrophy

Vitelliform macular dystrophy (VMD) is a slowly progressive macular dystrophy. Two forms of VMD with similar features have been described. The early-onset form (known as Best disease/Best macular dystrophy) is inherited in an autosomal dominant fashion and appears in childhood, while the adult-onset form with an uncertain inheritance pattern begins later in the mid-adulthood and tends to cause vision loss that worsens slowly over time [109, 89]. Best macular dystrophy (BMD) is one of the most common Mendelian macular dystrophies, occurring in about 1 in 10000 individuals; the prevalence of adult-onset form is not known.

Retinal findings are not generally present at birth and typically do not manifest until ages

5–10 years. Affected individuals have the characteristic lesion, a typical yellow yolk-like macular lesion on fundus examination. Over time, a pseudo-hypopyon appears due to gravitation of the yellow material inferiorly in the subretinal space, followed by deep and irregular pigmentation giving it a scrambled egg appearance. Lesions are usually bilateral but can be unilateral. Visual acuity is often 20/20 or better in eyes with undisturbed vitelliform lesions. Peripheral vision and dark adaptation remain normal. The prognosis is good till the fifth decade after which visual acuity declines in one or both eyes due to CNV, scarring, or geographic atrophy.

The diagnosis of BMD is based on characteristic fundus appearance, a light peak/dark trough ratio of less than 1.5 on EOG with a normal ERG and a family history of BMD. A normal EOG, however, does not exclude the possibility of BMD [17, 77, 88, 93, 149].

VMD is caused by mutations in the *BEST1* and *PRPH2* genes. *BEST1* mutations are responsible for BMD. The *BEST1* gene encodes for bestrophin protein that localizes to the basolateral membrane of RPE and appears to function as a calcium-sensitive chloride channel (Marmorstein AD et al). Mutations in the *BEST1* gene lead to impaired uptake of calcium across RPE leading to alterations in the adhesiveness between interphotoreceptor matrix and the RPE or diminution of outer segment phagocytosis, both of which are sensitive to the levels of calcium [87; 50]. In most of the cases of adult onset, the mutation is unknown, and less than a quarter have mutations traced to the *PRPH2* and *BEST1* gene. The *PRPH2* gene encodes peripherin 2, a protein essential for the normal functioning of photoreceptors, mutation of which causes vision loss by disrupting structures that contain light-sensing pigments [15, 36].

15.4.4 Congenital Stationary Night Blindness

Congenital stationary night blindness (CSNB) is a nonprogressive retinal condition of the scotopic vision. The prevalence of this condition is unknown. Two distinct phenotypes are recognized based on full-field ERGs, complete CSNB (CSNB1A-45%) and incomplete CSNB (CSNB2A-55%) [96, 97]. The complete and incomplete CSNBs are model disorders of bipolar cell dysfunction caused by dysfunction of on and off bipolar cells. Complete CSNB can be inherited in an X-linked recessive or autosomal recessive manner [96, 128]. The incomplete form appears to be more common in people of Dutch-German Mennonite descent [16]; however, it has been reported in families with many different ethnic backgrounds.

Mutations in the *NYX* and *CACNA1F* genes cause complete and incomplete forms of XL-CSNB, respectively. *NYX* and *CACNA1F* genes encode proteins that are specifically expressed in the retina: nyctalopin and voltage-dependent calcium channel for complete and incomplete CSNB, respectively [10, 135]. Mutations in these genes affect the synaptic transmission from photoreceptors (rods and cones) to the bipolar cells. In the complete form of XL-CSNB, the function of rods is severely disrupted and that of cones is only mildly affected. In the incomplete form, incomplete defect of synapses in the ON and OFF bipolar cells in both the rod and cone pathways occurs, but they do preserve the ability to detect the light [12, 98].

XL-CSNB is characterized by nonprogressive retinal findings with visual acuity ranging from 20/30 to 20/200 associated with refractive error, most typically myopia, but occasionally hyperopia, nystagmus, strabismus, normal color vision, and normal fundus examination [3, 16]. Individuals with CSNB1A generally report severe night blindness, while individuals with CSNB2A do not uniformly report severe night blindness. Other findings include defective dark adaptation; carriers of an *NYX* or *CACNA1F* mutation usually do not develop any of the visual problems related with XL-CSNB. However, carriers may have retinal changes detected with an ERG. Diagnosis is based on clinical findings, characteristic findings on ERG, family history, and molecular genetic testing of *NYX* and *CACNA1F*, the only two genes in which mutation is known to cause XL-CSNB.

15.4.4.1 Congenital Stationary Night Blindness Genetic Studies in India

A pilot study of eight complete CSNB cases from India identified mutations in all and in the candidate genes *TRPM1*, *GRM6*, and *GPR179* [85]. Further, using NGS-based targeted re-sequencing, a homozygous and a compound heterozygous mutations were identified in *SLC24A1* gene in two cases, confirming the inheritance pattern and genotype-phenotype correlation of autosomal recessive Rigg's type CSNB to this gene [106].

15.4.5 Leber Hereditary Optic Neuropathy

Leber hereditary optic neuropathy (LHON) is a rare form of vision loss associated with maternally inherited mitochondrial DNA mutations. The prevalence of LHON in most populations is unknown, and the relative frequency of the different LHON-causing mtDNA variants varies throughout the world. Overall, the m.11778G>A variant is the most prevalent, accounting for 70% of cases among northern European [83] and approximately 90% of cases in Asian populations [56, 90]. It affects 1 in 8500 to 1 in 50,000 people in northeast England and Finland, respectively [86, 112, 133].

LHON is characterized acute or subacute, severe painless unilateral loss of central vision during the young adult life. The fellow eye becomes subsequently affected within weeks or months of the first. Males are about four to five times more likely to be affected than females [160]. In the acute phase, the affected individuals are usually entirely asymptomatic until they develop visual blurring affecting the central visual field in one eye; similar symptoms appear in the other eye at an average of 2–3 months later. In about 25% of cases, visual loss is bilateral at onset. The ocular fundus may have a characteristic appearance that includes disc swelling, edema of the peripapillary nerve fiber layer, retinal telangiectasia, and increased vascular tortuosity. These changes can be subtle, and approximately 20% of the affected individuals show no fundal abnormalities. Visual acuity is severely reduced to counting fingers or worse in the majority of cases, and perimetry shows an enlarging dense central or centrocecal scotoma. Post-acute episode, the atrophic phase ensues in which the optic discs become atrophic within 6 weeks of onset. Significant improvements in visual acuity are rare, and in most individuals, vision remains severely impaired (Kirkman et al [67]). The hallmark of LHON is the selective degeneration of the retinal ganglion cell layer and optic nerve. The diagnosis of LHON is based on characteristic fundus findings in optic disc and vascular changes in the acute phase.

LHON is caused by mutations in the *MT-ND1*, *MT-ND4*, *MT-ND4L*, or *MT-ND6* genes. These genes are found in the mtDNA and encode subunits of NADH dehydrogenase. Mutations in any of these genes disrupt this process and lead to increased mitochondrial reactive oxygen species production that trigger retinal ganglion cell death via an apoptotic mechanism [11, 29, 162]. However, the selective vulnerability of retinal ganglion cells in LHON remains unexplained. A significant percentage of people with a mutation associated with LHON do not develop any features of the disorder. Specifically, more than 50% of males with a mutation and more than 85% of females with a mutation never experienced vision loss or related health problems.

15.4.5.1 Leber Hereditary Optic Neuropathy Genetic Studies in India

So far there are seven reports on LHON from India, screening either the primary mutations alone or the entire mitochondrial genome. The first report, a case study on two families identified MT:G3640A and MT:G11778A primary mutations, respectively [143]. Analyses of whole mitochondrial genome in a north Indian cohort of 30 cases and 20 controls revealed 6 pathogenic mutations including the primary mutations in 12 cases. The percentage of all variations, synonymous and non-synonymous, was higher in cases as compared to controls [70, 71]. A similar observation was seen in a study on 75 LHON cases and 40 controls from South India, wherein primary

mutations were identified in 27 cases and 4 other LHON-associated mutations as well. The above study also reports association of haplogroup M with LHON in the cohort [121]. In a study on 187 LHON families, 8 were positive for the primary mutation, MT:T14484C. A haplogroup analysis revealed that they belonged to diverse haplogroup contrary to the observed association of haplogroup J to MT:T14484C in the western population [64, 65]. A case report of two families with primary mutation, MT:G11778A along with MT:A1555G (usually associated with aminoglycoside-induced non-syndromic hearing loss), observed varying severity in the two cases but with no involvement of the auditory sense, thus suggesting role of nuclear modifying genes and environmental factors in the disease severity [64, 65]. Overall the frequency of LHON primary mutations observed in Indian cohorts is ~34%, whereas it contributes to >95% of the cases in western population [70, 71, 121, 138].

15.4.6 Fundus Albipunctatus

Fundus albipunctatus (FA), a flecked retinal disorder, is a subgroup of CSNB, characterized by nonprogressive night blindness and delayed dark adaptation, usually presenting in early childhood. The fundi show numerous small, yellow dots in the retinal pigment epithelium scattered throughout the fundus, which may or may not involve the macula [100]. The dark adaptation curve of affected individuals shows prolonged recovery of cone and rod sensitivity. The ERG cone and rod amplitudes are markedly reduced after 30–40 min of dark adaptation; however, they may come to normal or near-normal levels after many hours of adaptation.

FA is a genetically heterogeneous disorder with mutations in two genes, *PRPH2* (also known as RDS) and *RDH5* [47, 105, 156]. The *PRPH2* gene encodes peripherin 2, a protein involved in the formation and stability of the photoreceptors. The *RDH5* gene encodes enzyme 11-cis-retinol dehydrogenase that catalyzes the final step in the biosynthesis of 11-cis-retinaldehyde and transports it to the photoreceptors for incorporation into the photochromophore [14]. The inheritance pattern for families with mutations in *PRPH2* is consistent with autosomal dominant inheritance, while mutations in *RDH5* result in an autosomal recessive pattern. Mutations in *RLBP1* (retinaldehyde-binding protein 1) have also been found in some families.

15.4.7 Oguchi Disease

Oguchi disease is a CSNB characterized by the Mizuo-Nakamura phenomenon [95], a unique morphological and functional abnormality in which retina appears normal after prolonged dark adaptation, but on exposure to light, the retina displays a golden sheen with an unusually dark macula [20]. It was originally discovered in Japan where the prevalence is the highest but has been subsequently reported in European, American, Pakistani, and Indian patients [20, 21, 92, 145].

Individuals with Oguchi disease have a nonprogressive night blindness since young childhood with normal day vision, but they often claim improvement of light sensitivities when they remain for long time in a dark environment. The visual functions, including visual acuity, visual field, and color vision, are usually normal. On clinical examination, in addition to eliciting Mizuo-Nakamura phenomenon, they have a dark adaptation curve with a cone component but no rod-cone break and exhibit gradual recovery of full rod sensitivity after prolonged dark adaptation of 1–2 h. The clinical diagnosis is confirmed by genetic testing.

Oguchi disease is caused by mutations in the *SAG* (S-antigen, retina, and pineal gland) or *GRK1* (G-protein coupled receptor kinase 1) ([40]; Yamamoto et al. [155] Hayashi et al. [53]). The *SAG* gene codes for arrestin, a member of the rod phototransduction pathway (Oguchi type 1), while the *GRK1* gene codes for the rhodopsin kinase that works with arrestin in shutting off rhodopsin after it has been activated by a photon (Oguchi type 2). Some mutations in the *SAG* gene are associated with Oguchi disease and RP in the same family suggesting that mutations in *SAG* lead to RP.

15.4.7.1 Oguchi Disease Genetic Studies in India

Till date there are two publications on genetics of Oguchi disease from India. In a study on consanguineous family with three affected sisters, mapping with markers on chromosome 2q revealed linkage to a region between D2S172 and D2S345 [91]. In another report two affected sibs were identified with a nonsense mutation in codon 193 of the *SAG* gene [92].

15.4.8 Leber Congenital Amaurosis

Leber congenital amaurosis (LCA) is an early-onset childhood severe rod-cone dystrophy with a very poor prognosis. LCA occurs in 2–3 per 100,000 newborns being the commonest genetic cause of inherited blindness in childhood constituting more than 5% of all retinal dystrophies. It appears to be more prevalent where consanguinity is common [130].

Visual function is usually poor, accompanied by nystagmus, sluggish or near-absent pupillary responses, photophobia, high hyperopia, and keratoconus [26]. A characteristic finding is Franceschetti's oculo-digital sign, comprising eye poking, pressing, and rubbing [35]. The fundus appearance is extremely variable; the retina may initially appear normal, followed by pigmentary retinopathy reminiscent of retinitis pigmentosa observed later in childhood. The ERG is characteristically "non-detectable" or severely subnormal.

LCA has an autosomal recessive pattern of inheritance with more than half cases resulting from mutations in at least 17 different genes. These genes play a variety of roles in the normal development of the photoreceptors, phototransduction, and the functioning of cilia. Mutations in any of these genes disrupt the development and function of the retina, resulting in early vision loss. Mutations in *CEP290*, *CRB1*, *GUCY2D*, and *RPE65* genes are the most common causes of the disorder, mutations in other genes account for a smaller percentage of cases, and in about 30% of all people with LCA, the cause of the disorder is unknown [31]. Of note, three more specific retinal phenotypes can be observed: (a) preserved para-arteriole retinal pigment epithelium (PPRPE) in individuals with *CRB1* pathogenic variants; (b) "translucent RPE," white dots, and a peculiar star-shaped maculopathy in individuals with *RPE65* pathogenic variants; and (c) a progressive macular atrophic lesion presenting in infancy or later in some individuals. Because of its sharply defined borders, this lesion has been at times called a macular coloboma [23]. The diagnosis of LCA is established by clinical findings.

15.4.8.1 Leber Congenital Amaurosis Genetic Studies in India

There are very few reports on genetics of LCA from India. These studies have been either screening one or two candidate genes in a small cohort or participation in multicenter studies again with very few Indian subjects or identifying the candidate gene using homozygosity mapping. The screening of *RPE65* gene in cohort of 60 and 20 cases by Gandra et al. [81] and Ramana et al. [115] has identified a mutations in 1 and 3 cases, respectively. A study by Sundaresan et al. [137] to know the frequency of 104 mutations in 8 genes that contribute to 30% of LCA in northern American population showed that only 1 of the 38 case studies harbored a reported mutation. In another study on 30 LCA cases, wherein direct sequencing of RPE65 gene was followed by screening of reported pathogenic mutations using APEX technology, mutations were identified in 36% of the cases [144]. These reports indicate the genetic heterogeneity of the disease in our population and also involvement of novel variations in disease causation as reported in other ethnicities. Homozygosity mapping that has shown to map the candidate gene with >90% frequency was employed in a cohort of consanguineous LCA families, and in 11 of 12, the candidate gene and the causative variation were identified [134, 136, 137].

15.4.8.2 Therapies Under Investigation in LCA

In a naturally occurring Briard dog model of LCA resulting from mutation of *RPE65*, gene therapy utilizing AAV-mediated *RPE65* has been

shown to restore visual function, an effect that has been documented to last for more than 5 years [1]. The results of three simultaneous Phase I clinical treatment trials of AAV-mediated *RPE65* gene therapy in humans were recently reported [8, 24, 52, 84]. Initial results demonstrated safety and showed slight improvement in vision in both bright and dim light. Clinical and laboratory studies suggest that persons with *CEP290*-related LCA may also be good candidates for gene therapy. [24] studied the retinal architecture of *CEP290*-mutant mice and humans. In the mouse retina, dramatic retinal remodeling was evident by age 4–6 weeks. Cross-sectional imaging of affected human retinas performed using OCT indicated preservation of foveal cones. The relative sparing of foveal cone cells, despite severe visual dysfunction, suggests an opportunity for cell rescue.

15.4.9 X-linked Juvenile Retinoschisis

X-linked juvenile retinoschisis (XLJR) is a bilateral maculopathy with onset in the first decade of life. XLJR is inherited in X-linked recessive pattern. The prevalence of XLJR is estimated to be 1 in 5000 to 25,000 men worldwide [140]. Presentation is usually between the ages of 5 and 10 years; affected males generally present with reading difficulties. Visual acuity deteriorates during the first two decades and may remain stable until the fifth–sixth decades when further deterioration occurs due to progressive maculopathy [6]. Clinically, areas of schisis (splitting of the nerve fiber layer) in the macula giving the impression of a spoke wheel pattern may be visible. Schisis of the peripheral retina, predominantly inferotemporally, in approximately 50% of individuals has also been described [34].

XLJR progresses to retinal detachment in an estimated 5–22% of affected individuals. Retinal detachment can occur in infants with severe retinoschisis. About 4–40% of individuals with XLJR develop vitreous hemorrhage. The diagnosis of XLJR is based on fundus findings, electrophysiologic testing, and molecular genetic testing. *RS1* is the only gene known to be associated with XLJR. More than 196 pathogenic variants in *RS1* gene have been associated with XLJR [66]. *RS1* gene mutations result in a decrease or complete loss of functional retinoschisin, an extracellular protein that exists as a novel disulfide-linked octamer and is expected to play a crucial role in cellular organization of the retina [152]. Some individuals with X-linked juvenile retinoschisis do not have a mutation in the *RS1* gene. In these individuals, the cause of the disorder is unknown.

15.4.9.1 Therapies Under Investigation for X-linked Juvenile Retinoschisis

A mouse model of human X-linked juvenile retinoschisis was studied to determine whether supplementation with functional normal retinoschisin protein can produce improvement in ERG function and retina morphology [94, 164]. Subsequent evaluation of the mouse model confirmed that it appropriately mimics structural features of human X-linked juvenile retinoschisis. Replacements of the deficient protein through the use of a neomycin resistance cassette or through the use of an AAV vector were both successful, suggesting that, with additional study, gene therapy could become a viable strategy for therapeutic intervention [68].

Other researchers showed that older mice had significantly reduced benefit from AAV *Rs1h* cDNA (the mouse ortholog of human *RS1*) gene transfer compared to younger mice that had rescue of retinal structure and function. These benefits along with retinoschisin expression persisted for over 15 months. Other studies showed that intravitreal injection of a rAAV8 vector containing the mouse *Rs1h* cDNA under the control of a human retinoschisin promoter in *Rs1h* knockout mice yielded strong retinoschisin expression and structural and functional improvements. Wild-type retinoschisin delivered and expressed in human retinal culture cells has been shown to undergo protein folding, subunit assembly, and secretion mostly independent of endogenously expressed, defective retinoschisin protein. This suggests that gene therapy could be possible for

affected individuals who have residual, pathogenic RS1 protein expression [99].

Byrne et al. [19] explained that cell targeting and appropriate vector choice are very important to the success of retinal gene therapy. This group demonstrated that different cell types were able to secrete retinoschisin, transporting the protein across the retina. Photoreceptor cell secretion of this protein produced the best long-term rescue. Another therapeutic approach involves in vivo-directed evolution of AAV variants to deliver the wild-type gene to the outer retina after injection to the vitreous humor of the eye. The authors suggested that this has the potential to be a broadly applicable gene delivery method for inherited retinal diseases [28].

Possible drawbacks of using viral vectors (e.g., the risks of oncogenicity, immunogenicity, and the possible persistence of such vectors in the brain after intravitreal injection) have triggered an interest in non-viral systems. A combination of solid lipid nanoparticles, dextran, and protamine as well as EGFP and RS1 plasmids has been used to develop non-viral vectors for X-linked juvenile retinoschisis treatment. Researchers studied the in vitro transfection capacity, cellular uptake, and intracellular trafficking of these vectors in ARPE-19 cells. In vivo intravitreal, subretinal, and topical forms of vector administration in Wistar rat eyes were also evaluated. EGFP expression in various cell types depended on the administration route. This work suggests that these non-viral vectors may be useful in treating X-linked juvenile retinoschisis, other degenerative retinal diseases, and ocular surface diseases as well [30].

15.4.10 Norrie Disease

Norrie disease (ND) is an XL recessive neurological syndrome in which affected males are blind at birth or early infancy. It is caused by mutations in the *NDP* gene. ND is a rare disorder; its exact incidence is unknown [151]. It has been reported in all ethnic groups; although no ethnic group appears to predominate, most of the individuals reported in the first decades after the original description of Norrie disease were from Scandinavia [118].

The *NDP* gene encodes protein Norrin of the Wnt cascade which is essential for the specialization of retinal cells for their unique sensory capabilities. It is also involved in the establishment of blood supply to the tissues of retina and the inner ear and the development of other body systems. In order to initiate the Wnt cascade, norrin must bind to another protein called frizzled-4. Alteration in the norrin protein interferes with its ability to bind to frizzled-4, resulting in the signs and symptoms of Norrie disease [25, 79, 154].

Clinically ND is characterized by bilateral grayish yellow elevated mass (pseudogliomas) secondary to peripheral avascular retina, neovascular membranes, and tractional retinal detachments [146]. A child with ND unanimously presents with congenital blindness, and majority of them develop sensorineural hearing loss [108]. Almost half of the males with ND have developmental delay/intellectual disability or behavioral abnormalities. Though the general health is normal, the life span may be shortened secondary to risks associated with intellectual disability, blindness, and/or hearing loss, such as increased risk of trauma, aspiration pneumonia, and complications of seizure disorder [146]. The diagnosis of ND is based on the combination of clinical findings and molecular genetic testing of *NDP*.

15.4.11 Refsum Disease

Refsum disease is an inborn error of lipid metabolism characterized by a tetrad of retinitis pigmentosa, peripheral neuropathy, cerebellar ataxia, and elevated protein levels in the cerebrospinal fluid (CSF) without an increase in the number of cells. It is inherited in an autosomal recessive pattern. The prevalence of Refsum disease is unknown, although the condition is thought to be uncommon.

Refsum disease is characterized by anosmia and early-onset retinitis pigmentosa, both being universal findings with variable combinations of neuropathy, deafness, ataxia, and ichthyosis [131]. Onset of symptoms ranges from age

7 months to over 50 years. However, due to insidious onset, it is difficult to know exactly when the symptoms first started [131]. In general, individuals with retinitis pigmentosa due to Refsum disease keep some visual function until late in life although with severely concentrically constricted visual fields [119]. Early-onset disease is not essentially associated with a poor prognosis for life span. Cardiac arrhythmia and heart failure secondary to cardiomyopathy are severe health problems developing later in life. It should be noted that the full assemblage of signs and symptoms is rarely seen in an affected individual, and most of these features develop with age.

The diagnosis of Refsum disease requires analysis of phytanic acid concentration in plasma or serum followed by either molecular genetic testing or enzyme analysis. When present, normal plasma phytanic acid levels essentially rule out the Refsum disease. More than 90% of all cases of Refsum disease result from mutations in the *PHYH* gene that causes deficiency of phytanoyl-CoA hydroxylase; the rest are caused by mutations in a *PEX7* gene that lead to deficiency of the PTS2 receptor. Milder phenotypes caused by pathogenic variants in *PEX7* have also been reported [141]. Mutations in either the *PHYH* or *PEX7* gene disrupt the usual functions of peroxisomes, including the breakdown of phytanic acid, accumulation of which is toxic to cells, although it is unclear how an excess of this substance affects vision and smell and causes other specific features of Refsum disease.

15.4.11.1 Therapies Under Investigation

At present, the potential of enzyme replacement therapy (ERT) similar to that for lysosomal storage diseases (e.g., Hurler syndrome, Fabry disease, and Gaucher disease) is under investigation. This may eventually replace dietary restrictions and plasma- or lipapheresis. In the long run, gene therapy may be the treatment of choice, but many issues need to be resolved before it can be applied.

15.5 Syndromic RP

Sometimes RP occurs as part of syndromes in which other organs are affected. These conditions are called syndromic RP. Some of the syndromic RP are Usher syndrome, Bardet-Biedl syndrome (BBS), Refsum syndrome, Alstrom syndrome, etc.

15.5.1 Usher Syndrome

Usher syndrome is an inherited condition that is characterized by hearing loss and progressive loss of vision due to retinitis pigmentosa and, in some cases, due to cataract. Majority of patients retain some central vision throughout their lifetime [126]. Three types of Usher syndrome have been identified depending upon the severity of the disease and based on age of onset [62]. Type I, which is further subdivided into seven subtypes, is defined by complete deafness at birth or by the first year of life. Progressive vision loss and delayed sitting and walking due to problems with inner ear and thereby with balance are recognizable in childhood. While type II is identified through hearing loss from birth and visual impairment in adolescence or adulthood, type III is characterized by onset of clinical symptoms in adulthood.

About 3–6% of all childhood deafness and 50% of adult deaf-blindness are caused by Usher syndrome [126]. The estimated incidence of type I is about 4 in 100,000, while type II, rate of incidence unknown, is the most predominant form of the disorder. Type III accounts to only a small percentage of Usher syndrome except Finnish population [110]. Usher syndrome is an autosomal recessive disorder. Genes responsible for causing the disorder have been identified through linkage studies on families with affected individuals. Nearly 16 chromosomal locations and 13 genes have been recognized to cause Usher syndrome.

It is understood that Usher syndrome resembles cytoskeletal abnormalities, having a phenotype that occurs due to defective organization of microtubules that affects the stability and function

of photoreceptors and sensory cells in the inner ear. Mutations in *MYO7A*, *CDH23*, *PCDH15*, *USH1C*, *USH1G*, and *CIB2* genes are known to cause Usher syndrome type I (Keats), out of *MYO7A* gene has been reported predominantly in Usher syndrome type IB. Several homozygous missense and few deletion mutations have been identified in this gene. Experiments in mice models have shown the interaction of harmonin b, *CDH23*, and *MYO7A* genes which bring about proper shaping of hair bundles and cohesion of stereocilia [13]. About 35–39% of mutations causing Usher syndrome are observed in *MYO7A* and *CDH23* genes, and the R666X and IVS27-1G-C mutations constitute about 38% of mutations reported in that locus [107]. Riazuddin et al. [117] reported about 17 homozygous mutations in 23 Pakistani families.

Studies suggest that Usher syndrome types I and II follow similar pathogenic mechanism, in terms of the organization of hair bundles and their adhesion. A scaffold protein harmonin, *USH2A*, *VLGR1*, and *NBC3* genes are found to express in both photoreceptor and inner ear hair cells, and it is believed that the pathogenesis of type II is brought about by the interaction between these molecules. Mutations in *GPR98* and *USH2A* genes have been associated with Usher syndrome type II. More than 200 mutations have been identified in Usher syndrome type IIA. However a few mutations are known to cause only retinitis pigmentosa and not hearing loss, reason for which remains to be determined. Majority of the mutations detected are thought to result in shorter or no functional usherin protein; the mechanism underlying the pathogenesis is still unknown [2, 123]. Type III is the least common of the types of the syndrome recognized so far, except for Finnish population in which it accounts for about 40% of all cases of Usher syndrome. The clinical symptoms start in late childhood and with progression leading to profound impairment by middle age. Mutations in CLRN1 gene have been implicated in type III, among which the Tyr176Ter and Met120Lys mutations are common. The mechanism by the dysfunctional clarin 1 protein, which is thought to be the result of mutated gene, is not yet understood.

At least two gene therapy trials, aimed at restoring vision in patients, are underway: one on patients suffering from Usher syndrome type IB carrying *MYO7A* mutation and the other on those suffering from type II carrying a mutation in *USH2A* gene. While hearing loss can be treated to an extent by cochlear implant, the restoration of vision largely depends on the successful outcome of the trials that would inspire trials involving other gene mutations as well.

15.5.2 Bardet-Biedl syndrome (BBS)

BBS is a pleiotropic disorder characterized by clinical symptoms such as progressive RP, polydactyly, obesity, hypogonadism, genital anomalies, cognitive disabilities, and renal conditions [82]. Other minor features include delayed speech and development and dental anomalies.

Genetically heterogeneous in nature, Bardet-Biedl Syndrome (BBS) was first described by Lawrence and Moon when they reported a family with four siblings who presented with retinal dystrophy, obesity, spastic paraparesis, and mental retardation. Bardet and Biedl later described an additional phenotype associated with the syndrome, namely, polydactyly, and the term Lawrence Moon Bardet-Biedl syndrome came into existence. Several reports that followed suggested overlapping of the phenotypes, and currently it is known by the standard term BBS. It is a disorder characterized by ciliopathy in which eight proteins that assemble to form a BBSome complex involved in signal trafficking in cilia are implicated. BBS is an autosomal recessive disorder, and about 22 genes have been reported in BBS till now, namely, *BBS1*, *BBS2*, *BBS4*, *BBS5*, *BBS7*, *BBS9*, *BBS10*, *BBS12*, *ARL6*, *BBIP1*, *CEP290*, *IFT172*, *IFT27*, *INPP5E*, *KCNJ13*, *LZTFL1*, *MKKS*, *MKS1*, *NPHP1*, *SDCCAG8*, *TRIM32*, and *TTC8*. While BBS genes accounts for about 70–80% of the mutations reported in BBS so far, BBS1 gene is considered to contribute predominantly with about 40% of all the mutations reported.

Being an autosomal recessive disorder, homozygous mutations are commonly found. However

rarely triallelism is observed in BBS. Katsanis et al. [61] reported a BBS family in which two unaffected siblings carried a homozygous *BBS1* gene mutation, while affected sibling carried an additional heterozygous mutation in *BBS1* or *BBS6* gene. Epistatic modifier effects have also been observed in BBS. Patients who carried heterozygous mutations in *BBS2* and *BBS6* genes in addition to *BBS1* homozygous mutation have been observed to present severe and early-onset phenotypes compared to those who had only *BBS1* homozygous mutations [7]. Ciliopathy genes such as *MKS1*, *MKS3*, and *CEP290* genes have also been reported to exert epistatic effects on BBS gene mutations [161]. Although *BBS1* gene mutations predominate the overall mutations reported in BBS, they are also associated with milder phenotypes when compared to those reported in other BBS genes. Among the BBS genes, *BBS1*, *BBS2*, *BBS3*, and *BBS4* are frequently associated with ocular and digital phenotypes seen in BBS. Advanced technologies such as homozygosity mapping, exome sequencing, and next-generation sequencing have helped in the molecular analysis of mutations in the clinically and genetically highly heterogenic BBS phenotype. Although the impact of molecular diagnosis on therapy needs to be explored yet, their role in preventive and prenatal diagnosis is of importance.

To understand hereditary retinal dystrophies, extensive research has been attempted in animal sciences to characterize clinical, functional, structural, and molecular genetic studies in feline to create interventional strategies for genes like *CRX* and *CEP290* [102]. Interventional strategies in inherited retinal degenerative diseases have moved from huge barriers to prospective experimental successes through gene therapy, alternative pharmacological approaches, neuroprotection, optogenetics, and cellular therapy [120]. Therapeutic animal models for RP have shown that rhodopsin gene therapy-based augmentation and supplementation plus suppression are needed for the survival of the rods [78]. Interestingly, in optogenetics non-vertebrate light-sensitive molecules ChR2, VchR1, and NpHR derived from *Chlamydomonas reinhardtii*, *Volvox carteri*, and *Natronomonas pharaonis*, respectively, are used to stimulate the retina, paving way for preclinical studies soon [41]. Gene therapy is a good option in recessive diseases, whereas in dominant disorders, it may not work; hence, alternatively siRNA and microRNA pathways are explored, including using neurotrophic factors to stabilize the photoreceptor degeneration [42].

15.5.2.1 Bardet-Biedl Syndrome Genetic Studies in India

There is only one report on molecular genetics of Bardet-Biedl syndrome from India. A study on 30 families identified disease causative mutations in 24 (80%) of them, 22 in BBS candidate genes, and 2 in other ciliopathy gene, *ALMS1*. Of the identified variations, mutation in BBS3 gene including the novel recurrent mutation (p.I91T) accounted for 18% [124].

15.6 Conclusions

Gene mapping and characterization of genes causing inherited or familial ocular genetic disorders has been a priority and focus area in fighting blindness initiative. Extensive studies have been carried out in the western countries like the USA, Canada, the UK and Europe; however, there is need for more work to be carried out in countries in South Asia including India. Even in the whole of Asia, including Hong Kong, Singapore, Japan, China, and South Asia, all put together cannot match with the quantity and quality of work the West has done in ocular genetic studies, even though two-thirds of the population live in Asia with these diseases. There is a growing need for Asia and the West to network and perform more studies to identify all the genes that cause various ocular disorders which are genetic in nature both Mendelian and complex disorders.

Compliance with Ethical Requirements The paper is not a original work but a compilation of latest updates in the concerned field. Therefore, does not need ethical clearance.

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Unique Patient Populations in Asia for Genetic Eye Research

16

Himshikha Bhutani, Neel Kamal Sharma, and Akshay Anand

Abstract

DNA level similarities within the human race may have far-reaching consequences, but environment is a major factor determining the final outcome of genetics. It is therefore mostly agreed that individual population study should be an integral part of any major research programme. Asia is the most populated continent on Earth, and with the immense diversity that it holds, it is essential to identify unique patient populations for efficient genetic eye research. Consanguineous marriages are common in many Asian countries, and their consequences hold prevalence in the field of medical genetics. Novel ocular pathways have been suggested by ocular genetic conditions thus far unique to the region. Population-specific genetic intervention is important for better diagnosis and management of eye diseases. Each population is accompanied by a unique patient data set and needs elaborate and well divided study. Area wise description of patient population in Asia for genetic eye research has been provided in the following chapter.

Keywords

Population · Genetics · Asia · Eye diseases · Polymorphism

16.1 Introduction

It is mostly agreed that modern humans constitute a population which is quite young in its age, but the 'origin' of the modern human being remains a debatable issue. The scientific community is divided between supporters of the 'multiregional' hypotheses on one side and those supporting the 'Garden of Eden' hypotheses on the other side [1]. With the advent of modern genetic approaches, attention has been diverted towards extensive DNA level similarities among the human race, but major distinguishing features among groups like Asians, Australian aborigines and Europeans point towards uniqueness and may be brought about by environmental factors, which clearly demands individual population study [2].

Studies in ocular genetics opened the path to gene discovery on human chromosomes [3, 4].

H. Bhutani
Alumna, Neuroscience Research Laboratory, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

N. K. Sharma
Armed Forces Radiobiology Research Institute, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

A. Anand (✉)
Neuroscience Research Laboratory, Department of Neurology, Postgraduate Institute of Medical Education and Research, Chandigarh, India

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Currently, the burden of genetic diseases is on a rise, and it has been pointed out that routine ophthalmology clinics may not be sufficient to deal with this increasing burden of patients in need of simultaneous counselling and treatment [5]. Advances in molecular genetics have made diagnosis and prevention of certain eye diseases an accomplished reality [6]. Since heredity is finally a product of interaction between genetic and environmental factors, so the differences encountered at regional levels may stem from social practices pertaining to specific populations with each affecting evolution in its own way. Patient populations are thus structured and distributed in their own unique manner.

16.2 The Genetic Diversity of Asian Population

Asia is the largest continent on Earth and almost equally diverse. Cultural differences among Asians play a vital role in influencing gene flow which may be due to caste restrictions on marriages. An example of this is a study conducted in Southern India, wherein the researchers studied maternal and paternal lineages using the mitochondrial genome (mtDNA) and Y-chromosomal DNA, respectively, and confirmed that females in this population have a greater intercaste mobility as compared to men. It was found that the Y-chromosomal data did not correspond to social rank differences between castes, whereas mtDNA distances did [7]. The control region of mtDNA has also been studied in the Sindh region of Pakistan so as to generate data for population genetic epidemiological investigations [8]. The non-recombining feature of mtDNA and Y-chromosomal DNA has helped to establish sound evidence in support of a north-south divide within the East Asian population [9].

Founder events pertaining to specific populations play complex roles during the course of evolution and may result in higher rates of recessive diseases in these populations, for example, the 'Finnish founder mutations' [10]. Such events may also create population isolates which usually turn out to be excellent candidates for mapping both Mendelian and complex traits [11]. In the case of eye diseases, Mendelian inheritance pattern is normally followed by ocular conditions that have an early onset, whereas conditions appearing in adult life exhibit complex inheritance patterns. Data generated by an increasing number of studies is promoting genetic counselling prior to arranged marriages and prenatal testing of disease causing mutations. At the same time, as ocular genetics advances, ethical issues associated with such predictive testing are also cropping up [12].

Researchers have highlighted the need to study the evolutionary history of genetically diverse populations like those in Asian countries before designing further investigations for health and disease conditions [13]. The vision 2020 programme has managed to achieve impressive targets in combating major eye diseases that result in blindness [14], but such initiatives could reach their maximum potential if supported by population-specific genetic intervention for better diagnosis and management of eye diseases.

16.3 Genetic Eye Research and Asian Populations

Genetic eye research in complex eye diseases has recognized several susceptible genes, but result reproducibility becomes dependent on the population at hand. It is thus important to identify unique populations especially because of ancestry which also plays a major role in determining risk factors associated with diseases [15]. The importance of risk factors in eye diseases may be judged from an example of a study conducted on data pooled from across the major continents of the world, so as to assess the prevalence of retinal vein occlusion [RVO]. Hypertension is a risk factor associated with RVO, and since Asian and Hispanic populations have higher recorded cases of hypertension, consequently these two populations also reported a higher prevalence of branch retinal vein occlusion (BRVO) [16].

Starting from the colour of the eye, genes such as *OCA2* (oculocutaneous albinism II gene) and *HERC2* (HECT and RLD domain containing E3

ubiquitin protein ligase 2) have been studied with respect to country of origin to reveal that these genes face multiple selection pressures [17]. Ulivi et al. conducted a study to identify the genetics of eye colour across the ***Silk Road*** and they indicated towards a probability of genetic polymorphisms contributing to the phenotype in a manner specific to each population, by interacting with *OCA2* and *HERC2* genes [18]. Even if one considers the morphology of the eye, then comparisons within the broader Asian population are further followed by interethnic differences so much so that direct transferability of results is limited and clinical procedures have to be planned accordingly [19].

Wong et al. have pointed out the lack of precise and standardized Asian data required for a meta-analysis to be conducted in the area of eye diseases [20]. A lot of data, linking DNA sequence variation to diseases, is available from European studies, but it may lose its validity if applied in a parallel manner to Asian populations without further investigations [21, 22].

16.4 Population Division

16.4.1 West Asians

Buphthalmos or **primary congenital glaucoma** (PCG) is highly prevalent in **Saudi Arabia,** and since it follows an autosomal recessive pattern of inheritance, so a high frequency of occurrence is usually reported where consanguineous relationships are common. Mutations in genes coding for cytochrome P450, family 1, subfamily B, polypeptide 1 (*CYP1B1*) and latent-transforming growth factor beta-binding protein 2 (*LTBP2*) are most extensively studied in relation to this disease [23]. In the year 1997, Stoilov et al. first reported three different mutations in the *CYP1B1* gene linked to PCG in a **Turkish** population (**transcontinental Eurasia**) study [24]. The distribution of high variable *CYP1B1* mutations stands at 90–100% in Saudi Arabia and Gypsy population of Slovakia (Europe). In Saudi Arabia alone, 96% cases are linked to mutations in the *CYP1B1* gene [23, 25]. It may be noted that the disease shows a 100% penetrance in Turkish and Slovakia populations (possibly due to certain founder effects), whereas this penetrance rate is lesser for Saudi Arabian population [26].

Defining the age and racial categorization of a population is of utmost importance before discussing any results of such studies. As pointed out by Al-Mansouri et al., their study yielded a percentage prevalence of glaucoma at 1.73% in a **Qatar** population aged 40 years and above [27]. This figure is low compared to an **Oman**-based population study where the authors observed a prevalence of 4.75%. It is to be noted here that the age of population in the latter case was 30 years and above. Also, Oman hosts people of African origin, which might account for the higher prevalence [28].

Achromatopsia is an autosomal recessive disorder, and it mostly occurs due to mutations in any of these three genes: *CNGA3* (cyclic nucleotide-gated channel, alpha-3), *CNGB3* (cyclic nucleotide-gated channel, beta-3) and *GNAT2* (guanine nucleotide-binding protein, alpha-transducing activity polypeptide 2). In *CNGA3* gene, two mutations (Arg283Trp and Gly397Val) were found to be associated with this disease in two **United Arab Emirates**-based families. Large family sizes and endogamy are important factors taken into consideration for genetic eye research in these populations [29].

16.4.2 East Asians

East Asia is currently facing an epidemic with ever increasing cases of short-sightedness or myopia [30]. Studies examining the effect of genes and environmental factors on the development of refractive error are extensively available. A single nucleotide polymorphism (SNP) in the *CTNND2* (catenin delta 2) gene has been found to be associated with **high myopia** in **Chinese** and **Japanese** cohorts in **Singapore** [31]. Hammond et al., in a study conducted on a British female twin population, have underlined the importance of interpreting the results of such studies in a population-specific manner. So even though their results depict an 85% heritability of

spherical equivalence with genetic effects accounting for maximum population variance in a manner parallel to Finnish and Chinese twin studies nevertheless, environmental changes in a specific population hold strong at all times [32]. One such change could be urbanization of populations. For example, a study conducted by Pan et al. on a **multi-ethnic** Asian population within Singapore indicated a higher prevalence of myopia in the younger generation as compared to adults among all the three cohorts studied. This may be due to extreme changes in socio-environmental variables which accompany urbanization [33]. Similarly, a study conducted on a Japanese adult population from Tajimi city revealed a lower prevalence of blindness and low vision as compared to data from other parts of the world. The authors reasoned that this may be due to increasing public awareness coupled with a sound medical cover available to citizens irrespective of their economic background [34]. The impact of environment is so strong that it has been suggested that the prevalence of myopia among children depends less on their genetic lineage and more on the environment they grow up in [35]. Environment being a very broad concept needs geographical considerations so as to narrow down certain parameters, and two studies that deserve special mention within East Asia are the SiMES (Singapore Malay Eye Study) and the TPS (Tanjong Pagar Survey). Both these studies were based on data collected from subjects aged 40–79 years of age within Singapore [20]. While on one hand, the SiMES studied the Malay population of Singapore, on the other hand, the TPS studied the Chinese population of Singapore. The foundation behind conducting separate studies within the same geographical region highlights the importance of ethnic differences while studying eye diseases [36]. Ethnicity regulates the socio-environmental backdrop, which in turn decides the outcome of such studies.

Age-related macular degeneration (AMD) is a heritable disorder, and the elderly population is very likely to be affected by it. **Polypoidal choroidal vasculopathy (PCV)** is a subtype of exudative (Wet) AMD, and East Asians show a high predisposition towards this eye disease. Many loci have been identified to be playing a role in placing specific ethnic groups at a higher risk to develop the disease. A meta-analysis by Fan et al. utilized the previously identified 34 AMD loci [from the International AMD Genomics Consortium] to narrow down to 8 loci specifically associated with PCV. The authors also found a very high genetic correlation between PCV and typical neovascular AMD (tAMD), and certain risk alleles showed up more frequently in Asians as compared to Europeans [37].

Primary angle-closure glaucoma (PAC) is quite prevalent among East Asians. Anterior chamber depth (ACD) is a risk factor, but a single risk factor can never justify this high prevalence [38]. For **primary congenital glaucoma** (PCG), corresponding to data outlined above for West Asians, the distribution of high variable *CYP1B1* mutations stands at 15–20% among Japanese and Chinese populations [25].

The *OCA2* gene, mentioned in a previous section in relation with **eye pigmentation**, was studied by Murray et al. in East Asian populations, and their work revealed that out of the two known *OCA2* polymorphisms, the rs74653330 allele showed up almost exclusively in northern parts of East Asia, whereas the rs1800414 allele showed up frequently throughout the broader region of East Asia [39]. Overall, there is a need to establish a database that stores ethnic-specific genetic information for eye diseases because disease-associated mutations and even the phenotypic expressions of such genes may show ethnic variations [40].

16.4.3 South Asians

The untapped potential of gene mapping for health benefits in South Asian populations has been highlighted in a study by Nakatsuka et al. This study moves beyond the usual domain of consanguineous marriages of close relatives resulting in recessive diseases and instead focuses on founder events in a way parallel to founder groups recognized in European studies. In this way even non-consanguineous marriages get

included in the screening process for recessive disease-associated mutations resulting from recent shared ancestors [41].

The **Indian Genome Variation Consortium** sought to address, among many other questions, the issue of association between HapMap populations and Indian populations so as to better identify populations 'at-risk' of certain diseases. Though the study acknowledged a correspondence between these two populations, but it also documented various levels of genetic combinations based on ethnicity. The **rs1056827** allele of *CYP1B1* gene, discussed above for West and East Asian populations in relation with PCG, was identified as one of the 12 base SNPs valuable for categorizing populations of unidentified ethnicity as either large populations that are primarily caste based or tribal isolated populations with 100% precision. Within the entire geographical boundary of **India**, this allele showed an observed frequency ranging from 0.06 to 0.64 with central and east regions accounting for lowest frequency and western and south-eastern borders showing a very high frequency [42]. This gradual build-up of allele frequency differences is important in structuring populations. It has been noted that most alleles are pervasive in nature; therefore, genetic variation encountered in human populations are actually a result of varying degrees of frequencies of these alleles and not an isolated manifestation [43].

A study conducted by Gemmy Cheung et al. to compare a **central rural Indian population** with an **urban Indian population residing in Singapore** revealed an interesting outcome in **AMD** patients. Considering the vast difference between the environments encountered by these two populations, a corresponding difference in disease prevalence was not obtained. It was thus concluded that genetic factors may be more predominant in deciding the course of AMD as compared to environmental factors [44].

A study of *CNGA3* and *CNGB3* genes mentioned above for West Asians with respect to achromatopsia, revealed two unique mutations in two separate **Pakistani** families. These two mutations were firstly, a missense mutation in *CNGA3* gene which leads to replacement of a serine residue for an arginine residue (p.R274S) in the final protein product and secondly, a frame shift mutation in CNGB3 resulting in premature termination of the protein (p.V609WfsX9) [45].

16.5 Summary

Even as the controversy surrounding the exact origin of modern human beings continues, the need to study individual human populations keeps getting stronger. Genetic eye research has been pivotal in leading towards breakthrough discoveries in the field of genomics. These discoveries can positively impact patient populations if each of the data set is matched to ethnic and ancestral history of specific populations. Asia is multi-cultural and multi-ethnic in its societal background, and most of the people conform to a much conserved system of marriages. With such practices and also a high degree of recent shared ancestors, the genetic makeup of an average Asian may predispose the individual to various eye diseases. In order to tackle with the oncoming burden of such diseases, ophthalmology clinics need genetic counselling units equipped with sound knowledge of the unique patient populations at hand. We have thus attempted to describe patient population area wise, keeping genetic eye research at the forefront. Table 16.1 summarizes the information with regard to major populations discussed.

Table 16.1 Summarized representation of major populations discussed

Region	Eye disease	Associated gene	Population studied
West Asia	PCG	*CYP1B1*	Saudi Arabian
	Achromatopsia	*CNGA3* and *CNGB3*	UAE
East Asia	Myopia	*CTNND2*	Chinese and Japanese [in Singapore]
South Asia	Achromatopsia	*CNGA3* and *CNGB3*	Pakistani
	PCG	*CYP1B1*	Indian

Compliance with Ethical Requirements Himshikha Bhutani, Neel Kamal Sharma and Akshay Anand declare that they have no conflict of interest.

No human or animal studies were performed by the authors for this book chapter.

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Retina Genes in Chinese

17

Jingna He, Wai Kit Chu, Li Ma, Calvin C. P. Pang, and Guy L. J. Chen

Abstract

Retina is a multilayered structure containing several different cell types crucial for visual functions. Light signals are converted into electrochemical signals on the retina and transferred to the brain through the optic nerve. A dysfunctional retina leads to various retinal diseases that can result in vision impairment and even irreversible blindness. Genetic factors play important roles in retina structure and functions. In recent decades, there are significant advancements in mapping retina genes associated with retinal diseases, some are monogenic and some multifactorial in etiology. Some retinal diseases overlap in clinical courses and even genetic constitutions, with both similarities and differences in presentations among ethnic populations. We have investigated *CFH*, *HTRA1*, *BEST1*, *CETP*, *ABCA4*, *RHO*, *RP1*, *CYP4V2*, and other genes in Chinese patients with retinal diseases including age-related macular degeneration, polypoidal choroidal vasculopathy diabetic retinopathy, retinitis pigmentosa, Best vitelliform macular dystrophy, and Stargardt disease.

Keywords

Retina genes · Chinese · Retinal diseases

17.1 Introduction

Retina is a multilayered structure containing several different cell types. For the light to reach the rod and cone photoreceptors at the back of the retina, it has to pass through the ganglion cell layer and the layers of bipolar, Müller, and horizontal cells [1]. The outer segments of these photoreceptors contain pigments that absorb various wavelengths of the light and convert the light signal into electrochemical signals. These electrochemical signals are then transferred to the ganglion cells and eventually to the brain through the optic nerve [2]. Retina plays essential roles in vision. A dysfunctional retinal would lead to various retinal diseases, which could result in vision loss.

In recent decades, advent in high-throughput genomic technologies for large number of patient samples and vigorous studies in family pedigrees has led to identification of many retina genes that contribute directly or interactively with other factors to various retina genes. There has been sig-

J. He · W. K. Chu · L. Ma · C. C. P. Pang
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, China

G. L. J. Chen (✉)
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, China

Department of Ophthalmology and Visual Sciences, Prince of Wales Hospital, Hong Kong, China
e-mail: lijia_chen@cuhk.edu.hk

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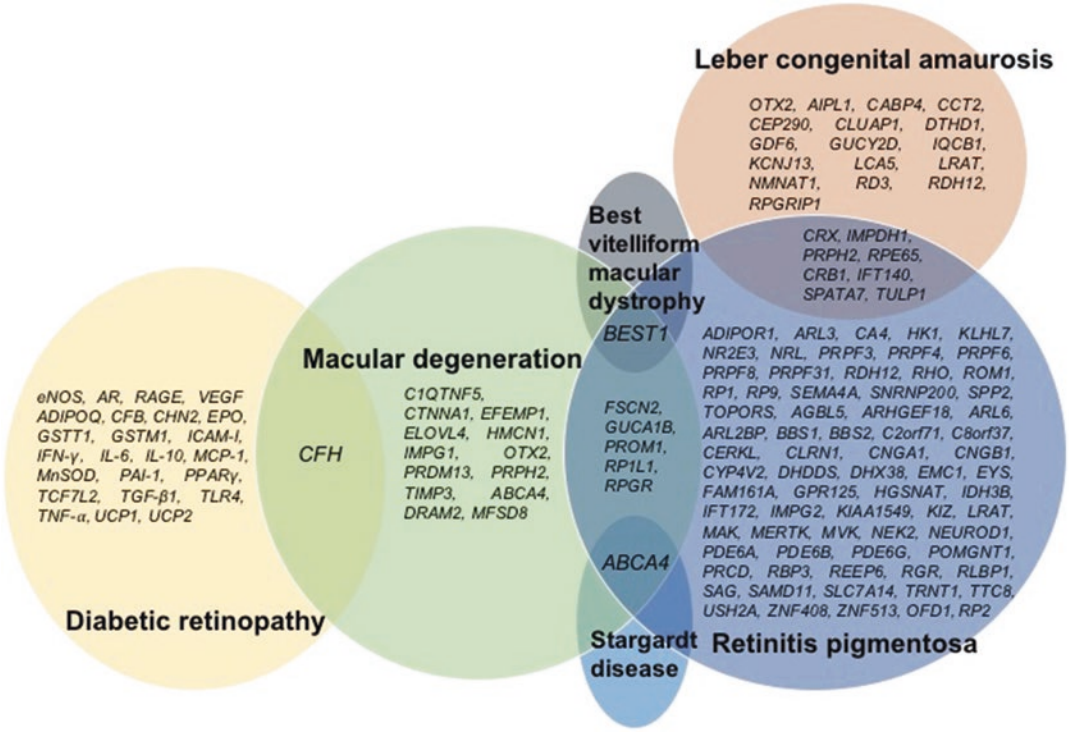

Fig. 17.1 Mapped and identified genes in major retinal diseases

nificant advancement in understanding the contribution of both genetic and environmental factors (Fig. 17.1). While properties of some retina genes have helped to understand disease mechanism and thus aid in development of effective treatment modes, more understanding is still needed to delineate pathophysiology of many potentially blinding retinal diseases, especially those with complex etiology. In this chapter, the genetic and environmental factors of retinal diseases that occur in the Chinese population will be reviewed. Some of them are monogenic diseases, and some are multifactorial in etiology.

17.2 Monogenic Retina Diseases

17.2.1 Retinitis Pigmentosa (RP)

Retinitis pigmentosa (RP) is a group of hereditary retinal diseases that feature degeneration of rod and cone photoreceptors [3]. In most RP patients, rod cells would degenerate before the cones. These patients usually lose their night vision initially in adolescence, followed by losing side vision in young adulthood and central vision at older ages [3]. Usually RP is a disease that is confined to the eyes. But about 20–30% RP patients are associated with complex diseases such as Usher syndrome and Bardet-Biedl syndrome (BBS), which associate with hearing impairment and ciliopathy, respectively [3]. The prevalence of RP is around 1 in 4000 worldwide. More than one million people are affected by RP [3]. Inheritance patterns of RP are mainly autosomal dominant (30–40% of total cases), autosomal recessive (50–60%), and X-linked (5–15%) RP [3]. In China, the prevalence of RP is very similar to the global data, which was about 0.03% (1 in 3784) in Eastern, mid-Southern, North-Western, and Northern China, based on a mass screening of 196,777 people [4]. In 2006, a population-based, cross-sectional cohort study was conducted for 4439 study subjects aged older than 40 years. The prevalence of RP showing pathological fundus appearances and visual function losses was about 1 out of 1000 elderly Chinese in Northern China, which could be

extrapolated to around 1.3 million patients based on the total population in that area [5].

RP is essentially a monogenic disorder inherited as Mendelian trait. At least 89 RP-associating genes have been mapped (https://sph.uth.edu/retnet/sum-dis.htm#B-diseases). *Rhodopsin* (*RHO*) was identified in RP in 1990 [6]. Most RP genes are only involved in only one form of Mendelian inheritance (autosomal dominant or recessive), while a few genes such as *NRL*, *RP1*, and *RHO* could be inherited in both forms. Including all the mutations found to cause non-syndromic RP, about 3100 disease-causing mutations have been reported in the Human Gene Mutation Database (HGMD). Another 1200 mutations have been reported to cause Usher syndrome and BBS [7].

In autosomal dominant RP, 28 loci have been identified. Mutations in most of these genes account for a small proportion of RP cases except the *RHO* on 3q22.1, which causes around 25% of autosomal dominant RP [3]. A sequencing study on all coding regions and adjacent intronic regions of *RHO* in 248 Chinese probands with non-X-linked RP detected eight heterozygous nucleotide changes in *RHO*, c.628G>T, p.Val210Phe; c.945C>G, p.Asn315Lys; c.527C>T, p.Ser176Phe; c.568G>T, p.Asp190Tyr; c.768_770delCAT, p.Ile256del; c.1040C>T, p.Pro347Leu; c.310G>A, p.Val104Ile; and c.895G>T, p.Ala299Ser [8]. In Table 17.1, we summarize *RHO* variations in RP of Chinese population-based studies.

To date 61 gene loci have been mapped in autosomal recessive RP, and about 20% of recessive RP are caused by mutations in *USH2A* [3]. In the Chinese population, many mutations in *USH2A* have been determined (Table 17.2). They would be useful for the molecular diagnosis and disease management of Usher syndrome. Comparing with autosomal dominant and recessive RP, less genes have been mapped for X-linked RP. There are six of them: *OFD1*, *RP2*, *RPGR*, *RP6*, *RP24*, and *RP34*. *RPGR* accounts for 70% of X-linked RP [3]. In the Chinese population, several *RPGR* mutations have been found (Table 17.3).

17.2.2 Leber Congenital Amaurosis (LCA)

Theodor Leber, a German ophthalmologist, first described Leber congenital amaurosis (LCA) in 1896, which is now used to describe a group of

Table 17.1 Sequence variations detected in *RHO* of RP patients in Chinese

DNA sequence variations	Amino acid changes	References
c.527C > T	p.Ser176Phe	[8]
c.568G > T	p.Asp190Tyr	
c.628G > T	p.Val210Phe	
c.768_770delCAT	p.Ile256del	
c.945C > G	p.Asn315Lys	
c.1040C > T	p.Pro347Leu	
c.310G > A	p.Val104Ile	
c.895G > T	p.Ala299Ser	
c.-300_-302delTTT	No amino acid change	[9]
c.-201C > T	No amino acid change	
c.625G > A	p.Val209Met	
c.891C > T	p.Ser297Ser	
c.155C > T	p.Arg21Cys	[10]
c.423G > C	p.Cys110Ser	
c.639G > T	p.Gly182Val	
c.653 T > G	p.Cys187Gly	
c.369C > T	p.Thr92Ile	
c.627A > G	p.Tyr178Cys	
c.409_426delGTGGTGGTGTGTAAGCCC	No amino acid change	

Table 17.2 Sequence variations detected in *USH2A* of RP patients in Chinese

DNA sequence variations	Amino acid changes	References
c.4384delA	p.Thr1462Leufs*2	[11]
IVS47 + 1G > A	Splice site	
c.13156A > T	p.Ile4386Phe	
c.8559-2A > G	No amino acid change	[12]
c.8272G > T	p.Glu2758X	[13]
c.12376_12378ACT > TAA	p.Thr4126X	
c.6875_6876insG	p.Arg2292ArgfsX39	[14]
c.8284C > G	p.Pro2762Ala	[15]
c.9958G > T	p.Gly3320Cys	
c.14287G > C	p.Gly4763Arg	
c.8559-2 T > C	No amino acid change	
c.11235C > G	p.Tyr3745X	
c.2802 T > G	p.Cys934Trp	[16]
c.8232G > C	p.Trp2744Cys	
c.1876C > T	p.Arg626X	
c.6249delT	p.Ile2084fs	
c.3788G > A	p.Trp1263X	
c.9492_9498delTGATGAT	p.Asp3165fs	
c.7123delG	p.Asp3165fs	
c.14403C > G	p.Asp3165fs	
c.5200G > C	p.Gly1734Arg	[17]
IVS32 + 1G > A	No amino acid change	

Table 17.3 Sequence variations detected in *RPGR* of RP patients in Chinese

DNA sequence variations	Amino acid changes	References
c.1059 + 1 G > T	No amino acid change	[18]
c.2002dupC	p.His668PfsX4	
c.2236_2237delCT	p.Glu746RfsX22	
c.2899delG	p.Phe967LfsX121	
c.2417_2418insG	p.Glu806fs	[19]
c.2233_34delAG	p.Glu746RfsX22	[20]
c.2236_2237delGA	p.Glu746RfsX22	
c.2403_2404delAG	p.Glu802GfsX31	
c.851C > G	No amino acid change	
c.2260G > T	No amino acid change	
g.ORF15 + 556delA	p.Lys184fs	[21]
g.ORF15 + 483_484delGA	p.Glu746ArgfsX768	[22]
g.ORF15 + 652_653delAG	p.Flu802GlyfsX833	
g.ORF15 + 650_653delAGAG	p.Thr801ThrfsX813	

retinal dystrophy causing blindness before the age of 1 year. LCA is a congenital disease associating with poor pupil responses, nystagmus, and a severely subnormal or undetectable full-field electroretinogram (ERG) [23]. It affects 1 in 33,000 newborns worldwide and 1 in 81,000 in North America. LCA represents around 5% of all inherited retinal dystrophies and 20% of children with visual impairment around the world [24]. In the Chinese population, the prevalence of LCA is not known. LCA is genetically heterogeneous. To date, mutations in more than 25 genes have been identified in LAC patients. Only one gene, *CRX*, involves in both autosomal dominant and reces-

sive forms. There are 3 genes inherited in autosomal dominant trait and 23 genes autosomal recessive. The three frequently mutated LCA genes are *CEP290*, *GUCY2D*, and *CRB1* [25].

In a Han Chinese cohort, screening of variants in 15 genes in 87 unrelated LCA patients identified 35 pathogenic mutations, which 66% of all detected variants. In these Chinese patients, variants in *GUCY2D* are the most common causes of LCA (16.1% of study patients), followed by *CRB1* (11.5%), *RPGRIP1* (8%), *RPE65* (5.7%), *SPATA7* (4.6%), *CEP290* (4.6%), *CRX* (3.4%), *LCA5* (2.3%), *MERTK* (2.3%), *AIPL1* (1.1%), and *RDH12* (1.1%). The prevalence is different from other populations. Three mutations in *GUCY2D* gene have been detected in this study, c.164C>T, c.935C>T, and c.2302C>T [26]. Variations in the *GUCY2D* gene causes 10–20% of recessive LCA and up to 40% of dominant corn dystrophy or corn-rod dystrophy [27]. *GUCY2D* codes for the retinal-specific guanylate cyclase 1, which localizes in the outer segment of the photoreceptors. It is involved in the phototransduction recovery process for cGMP resynthesis.

Whole exome sequencing (WES) of 41 Chinese LCA families found 17 variants in 19 genes [28]. *GUCY2D*, *CRB1*, *RPGRIP1*, *CEP290*, and *CRX* were the five most frequently mutated genes, consistent with results of an earlier study [26]. In another study on Chinese LCA families, direct Sanger sequencing of 7 genes (*AIPL1*, *CRB1*, *CRX*, *GUCY2D*, *LRAT*, *RDH12*, *RPE65*) in 117 LCA families identified homozygous or compound heterozygous mutations in 107 families. Heterozygous autosomal dominant mutations were identified in three families and an X-linked mutation in one family. In total, 136 mutations were found [29].

17.2.3 Best Vitelliform Macular Dystrophy

Best vitelliform macular dystrophy (BVMD) was first described by a physician Friedrich Best in 1905. BVMD is commonly inherited in autosomal dominant form. It is a progressive and juvenile-onset macular degeneration. In the subretinal and sub-RPE spaces, there are large deposits of yellow pigmented materials in BVMD patients, giving a typical yolk-like macular lesion on fundus photo. The appearance of the lesion looks like egg yolk, and then it would develop into a pseudohypopyon stage resulting in detachment of the neural retina from the RPE layer. The deposits would become disorganized with pigment accumulation, causing an increased thickness of RPE/choroid layer. A thinner retinal layer is also observed following the photoreceptor degeneration and central vision deterioration [30]. BVMD is a rare disease. Prevalence was 2 in 10,000 in Sweden, 1.5 in 100,000 in Denmark, and between 1 in 16,500 and 1 in 21,000 in a US study [31].

Mutations in the *BEST1* gene, also called *VMD2*, were initially found associated with BVMD. It is located on chromosome 11q13 and encodes the BEST1 protein, mainly expressed in RPE, but also in the testis, placenta, and brain. There are 585 amino acids in human BEST1, the first 350 amino acids conserved in different species. BEST1 works as an anion channel and a regulator of intracellular calcium signalling in RPE [31]. To date, five retinal degeneration diseases have reported associations with *BEST1* mutations. They are Best vitelliform macular dystrophy, autosomal recessive bestrophinopathy, adult-onset vitelliform macular dystrophy, autosomal dominant vitreoretinochoroidopathy, and retinitis pigmentosa. To date, 269 mutations have been reported in the *BEST1* gene across different ethnicities (http://www.huge.uniregensburg.de/BEST1_database/variants.php?action=search_unique&select_db=BEST1). In the Chinese population, we have directly sequenced of *BEST1* in 27 subjects from 7 Chinese BVMD families and 100 unrelated healthy Chinese subjects. Each BVMD family was found to have a unique mutation. We identified seven missense mutations (p.Thr2Asn, p.Leu75Phe, p.Ser144Asn, p.Arg255Trp, p.Pro297Thr, p.Asp301Gly, p.Arg218Cys). Two of these six novel mutations are located within the four common mutation clusters within the *BEST1* gene [32].

Five other studies screened *BEST1* mutations in Chinese. In 13 patients of 12 unrelated Chinese families affected by BVMD, 5 mutations (p.T4I, p.A291V, p.R218C, p.Q293H, and p.D301G) were identified [33]. In another study direct sequencing of *BEST1* in a 10-year-old patient, his family members and 200 unrelated subjects revealed heterozygous mutations c.292G>A (p. Glu98Lys) in exon 4 and c.1608C>T (p. Thr536Thr) in exon 10. These two mutations were not found in any of the patient's unaffected family members or in the normal controls [34]. Liu et al. identified six mutations (p.Ser16Phe, p.Ser144Asn, p.Glu292Lys, p.Glu300Lys, p. Thr307Asp, p.Arg47His) from 13 BVMD individuals from 6 unrelated families [35]. Recently, Tian et al. identified 36 disease-associating variants in *BEST1*, including 28 (77.8%) missense, 3 (8.3%) nonsense, 4 (11.1%) splicing, and 1 (2.8%) frameshift mutations from 17 BVMD patients and 20 autosomal recessive bestrophinopathy (ARB) patients. Results of these studies showed the spectrum of *BEST1* mutations in Chinese patients is different from that of Caucasian patients [36].

17.2.4 Stargardt Disease

Stargardt disease (STGD) was first described by Karl Stargardt in 1909, representing a set of juvenile macular degeneration diseases. STGD is typically inherited in an autosomal recessive trait. The prevalence of STGD is 1 in 10,000 [37] and is characterized with yellowish flecks around the macula and degeneration of RPE and the neural retina layer. Patients with STGD are affected bilaterally, with gradual decline in vision between 6 and 20 years old. These patients usually have no prior history of abnormal visual acuity [37].

All recessively inherited cases of STGD are due to mutations in the photoreceptor-specific ATP-binding cassette transporter gene *ABCA4* (formerly known as *ABCR*), which encodes the ABCR protein. ABCA4 acts as a flippase for N-retinylidene-phosphatidylethanolamine (PE) to facilitate the transport of all-trans retinaldehyde from intradiscal space into the cytoplasm of the photoreceptor outer segment. Mutations in *ABCA4* also contribute to other retinal diseases including retinitis pigmentosa and age-related macular degeneration (AMD) [38].

We also identified mutations in *ABCA4* gene in our Hong Kong Chinese cohort. We genotyped 140 AMD, 18 STGD patients, and 95 normal control subjects for 15 *ABCA4* exons. In STGD, p. Arg2040Stop was detected in two patients but not in controls. Thus it is likely a disease-causing mutation. Another sequence alteration p. Thr1428Met was found in 2 STGD patients, 18 AMD patients, and 15 normal people, implying Thr1428Met could be a polymorphism [39].

17.3 Multifactorial Retina Diseases

17.3.1 Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a progressive chronic retinal disease and a leading cause of vision loss in the elderly population worldwide [40]. There are two principal forms of AMD, exudative AMD, also called wet AMD, and geographic atrophy (GA), also referred to as dry AMD. In exudative AMD, fibrous scarring often happens after leaking of fluid, blood, and lipid from the retinal blood vessels as choroidal neovascularization penetrates into the neural retina. AMD was considered untreatable until photodynamic therapy was available [40]. In 2006 a landmark clinical trial showed that monthly intravitreal injections of ranibizumab, an antibody inhibiting a protein vascular endothelial growth factor (VEGF), could prevent vision loss in nearly 95% of AMD patients [41, 42]. A similar antibody, bevacizumab was also developed and could be supplied at a lower cost with comparable efficacy to ranibizumab [43]. There have been many epidemiological studies on AMD over the years worldwide. It is notable that the proportion of blindness caused by AMD in East Asian populations has increased from 5% in 1990 to 6.9% in 2010. It is now the third leading cause of blindness in East Asia [44]. By 2040, Asia is predicted

to contribute to the highest population of AMD patients in the world [45].

Strong evidence of genetic influences on the development of AMD has been given by familial aggregation, segregation, linkage, and twin studies. Since 2005, genetic loci have been reported to be associated with AMD as a result of GWAS. The complement factor H (*CFH*) gene on 1q32 was reported for dry AMD and the *ARMS2*/*HTRA1* locus on the 10q26 gene cluster for wet AMD with Hong Kong Chinese as the primary study cohort [46–49]. In Chinese study subjects, we have found higher HTRA1 protein expression in human vitreous with *HTRA1* rs2672598 C alleles. But rs11200638 was not correlated with HTRA1 level in human vitreous [50]. Sequencing of HTRA1 revealed higher prevalence of the c.34delCinsTCCT allele in controls (8.0%) than in nAMD patients (1.9%) [50]. In cultured retina pigment epithelial cells (ARPE-19), the recombinant HTRA1 c.34delCinsTCCT variant protein was more localized in the endoplasmic reticulum of RPE cells compared with the wild-type protein, and its secretion was delayed. ARPE-19 cells expressing HTRA1 c.34delCinsTCCT variant had higher cell viability, lower cell apoptosis, and less response to anoikis, supporting its protective role [51].

Associations of AMD with *C2*, *CFB*, *C3*, and *CFI* in the complement pathway have also been reported [46, 52, 53]. Several genes in the HDL cholesterol pathway, *LIPC*, *CETP*, *ABCA1*, and *LPL,* have been reported to associate with AMD. Apolipoprotein E (*ApoE*) has been suggested to be associated with AMD in the Dutch population, but our study in Chinese did not show association. Other genes like *COL10A1*, *COL8A1*, *TIMP3*, and *VEGFA* have also been associated with AMD [40].

Genome-wide association study (GWAS) and exome-wide association study (EWAS) showed 9 out of the 21 loci in East Asia that are strongly associated with AMD in European populations. These loci are *ARMS2/HTRA1* rs10490924, *CFH* rs10737680, *CETP* rs3764261, *ADAMTS9* rs6795735, *C2-CFB* rs429608, *CFI* rs4698775, *TGFBR1* rs334353, *APOE* rs4420638, and *VEGFA* rs943080. In addition, four other foci were found, and especially *CEPT* p.Asp442Gly (rs2303790) is specific to the East Asian population which is strongly associated with exudative AMD with an odd ratio of 1.70 [54].

AMD is a multifactorial disease with complex etiology involving genetic and environmental factors. In Western populations, risk factors for AMD older age are the major risk for AMD, with more than 10% of people older than 80 years having late AMD [55]. Dark iris pigmentation, previous cataract surgery, and hyperopic refraction are ocular risk factors for AMD [56–58]. Systemic risk factors including cigarette smoking, obesity, sunlight exposure, and cardiovascular diseases have been found in AMD. Smoking is a strong and most consistent risk factor for AMD [59]. In Asian AMD, age is also the major risk factor for Asian AMD and cigarette smoking the most consistent systemic risk factor [60–62]. In studies on Asian populations, other demographic and systemic risk factors have been reported, including male gender, hypertension, hyperlipidemia, high levels of high-density lipoprotein (HDL), chronic kidney disease (CKD), hepatitis V surface antigen, liver cancer, coronary heart disease, lower education levels, and increased serum white blood cell counts [63]. Both genetic susceptibility and environmental factors are important determinants to the onset and progression of AMD.

17.3.2 Polypoidal Choroidal Vasculopathy (PCV)

PCV is a vascular disease in the choroid. It occurs more commonly in Asian than Western populations [64–68]. In GA, the retinal pigment epithelium (RPE), choriocapillaris, and photoreceptors show cumulative atrophy, which lead to retinal degeneration. Unlike AMD, there is less epidemiological data about PCV. It is difficult to obtain accurate estimation of PCV prevalence based on a population-based screening with only fundus photos. Fluorescein angiography and indocyanine green (ICG) staining are required to diagnose PCV [69]. Hospital- or clinic-based cross-sectional studies have been conducted to estimate prevalence of PCV. In Asia, 22.3%–

61.6% prevalence has been reported, compared to 8%–13% prevalence in Caucasian [70–73].

A systematic review on the genetic associations of PCV with 56 polymorphisms in 19 genes/loci [83] revealed polymorphisms in 5 genes/loci that are involved in the complement pathway (*CFH* p.Try402His, rs1061170; *CFH* p.Ile62Val, rs800292; *C2*, rs547154; *CFB*, rs4151657; *RDBP*, rs3880457; and *SKIV2L*, rs2075702 and rs429608). Two genes/loci are related to the inflammatory pathway (*TNFRSF10A-LOC389641*, rs13278062 and *BEST-C4orf14-POLR2B-IGFBP7*, rs1713985). Extracellular matrix/basement membrane regulatory pathway (*ARMS2* p.A69S rs10490924 and *HTRA1* promoter rs11200638) and lipid metabolism pathway (*CETP* rs3764261) were also reported to be associated with PCV [63].

In the Hong Kong Chinese population, we identified specific genetic associations in AMD and PCV in Chinese. We found angiopoietin 2 (*ANGPT2)* a susceptibility gene for PCV and nAMD [74]. Two haplotype-tagging SNPs rs4455855 and rs13269021 in *ANGPT2* associated with PCV and nAMD in Chinese and Japanese [74]. Angiopoietin 2 is elevated in aqueous of patients with nAMD [75]. We reported the first association of placental growth factor (*PGF*) rs2268615 and rs2268614 with exudative AMD [76]. The first exome sequencing study on PCV utilized Chinese patients as primary cohort and identified a rare c.986A>G (p.Lys329Arg) variant in the *FGD6* gene that conferred risks to PCV, but not to AMD [77]. This is the first gene identified for PCV.

We conducted a series of genotyping studies on PCV and neovascularized AMD in the Chinese population. Our data showed *SKIV2L* is a susceptibility gene for neovascular AMD, independent of *CFH* and *HTRA1,* but not for PCV [78]. For genes in the complement pathway, *C3*, SNP rs17030, is not associated with neovascular AMD but with PCV, though only in males but not females [79]. Serpin peptidase inhibitor, clade G, member 1 (*SERPING1*) gene associated with AMD or PCV in Caucasians but not in Chinese or Japanese, indicating ethnic diversity in the genetic etiology [80]. We have found *ARMS2* SNP rs10490924 and *HTRA1* SNP rs11200638 conferred greater risks to AMD than to PCV [81]. In the high-density lipoprotein (HDL) metabolic pathway, *CETP* is a susceptibility gene for neovascular AMD and PCV independent of CFH and HTRA1 [82]. According to genotype studies in our Chinese cohort with neovascular AMD and PCV, there are consistent associations of the *CFH*, *ARMS2/HTRA1*, and *CETP* genes, indicating sharing of partially similar molecular mechanisms. However, the different genetic effects from the complement pathways also show existence of additional genetic and environmental factors affecting them to different extents [83].

17.3.3 Diabetic Retinopathy

Diabetes mellitus (DM) is one of the fastest growing chronic diseases globally. Various complications in DM can be grouped into macrovascular and microvascular complications. Diabetic retinopathy (DR) belongs to the microvascular complication of DM and is a leading cause of vision loss in middle-aged working population [84]. Thirty percent of patients with DM would result in DR, and 5–10% of them may develop into proliferative DR. Severe DR affect patients physically and emotionally, costing heavy healthcare-related resources [84].

There are proliferative and nonproliferative forms of DR according to the growth of the new retinal blood vessels. In patients having mild and nonproliferative DR, if left untreated, the retinopathy could progress into moderate or severe nonproliferative DR or even to proliferative DR. In the proliferative stage, abnormal new retinal blood vessels would appear. In some DR patients, diabetic macular edema (DME) may also happen [85].

Recent clinical trials showed that the progression of DR can be reduced by controlling blood glucose level, timely laser retinal photocoagulation treatment, and intraocular administration of anti-vascular endothelial growth factor (anti-VEGF) agents. According to World Health Organization (WHO), DR accounts for 4.8% (37 million people) of blindness worldwide [86].

There are approximately 93 million people with DR worldwide, 17 million with proliferative DR, 21 million with diabetic macular edema, and 28 million with vision-threatening DR [87]. In Chinese, DR prevalence among the type 1 DM was reported to be 14% [88]. In rural areas of China, the overall prevalence of type 2 DR has been reported to be 43.1% and prevalence of proliferative DR, macular edema, and vision-threatening retinopathy 1.6%, 5.2%, and 6.3%, respectively [89]. A study in urban areas of China found the prevalence of DR among adult diabetic Chinese was about 27.9% [90], which was much lower than that in the rural area.

DR is complex in clinical manifestations and in genetics. Twin studies, family studies, candidate gene studies, linkage studies, and GWAS had reported *VEGF*, receptor for advanced glycation end products (*RAGE*), endothelial nitric oxide synthase (*eNOS*), and aldose reductase (*AR*) to be involved in the pathogenesis of DM or DR.

In the *VEGF* gene, several polymorphisms have been reported: rs833061 (−460T/C), rs699947 (−2578C/A), rs2010963 [(405G/C) and (634G/C)], and rs3025039 (+936C/T). In the Chinese population, -460C/T was also correlated with nonproliferative DR [91]. Significant associations of DR were reported for SNP rs699947, rs833061, rs13207351, and rs2146323 [92]. Yang et al. suggested that polymorphisms in the promoter region of the *VEGF* would increase the risk of DR in Chinese patients with type 2 diabetes mellitus (T2DM) [93].

eNOS, *RAGE*, and *AR* are not well reported in the Chinese population. The function of eNOS protein is to regulate vascular tone by inhibiting smooth muscle contraction and platelet aggregation. There was no significant association between *eNOS*-4b/a polymorphism and DR in patients with type 2 diabetes mellitus [94, 95]. RAGE protein regulates oxidative stress and endothelial function in T2DM. The p.G82S polymorphism in RAGE was associated with DR in one reported Chinese study [96]. AR converts glucose to sorbitol in the polyol pathway. A meta-analysis showed significantly increased risks for DR in association with *AR* -106C/T variants in Chinese Han population [97].

IL-6 genotype of rs1800795 GC and rs1800796 GG might affect risk for type 2 DM patients suffering from proliferative DR in a Chinese study [98]. Association of rs507392, rs1617640, and rs551238 minor alleles of erythropoietin with increased DR risk has been reported [99]. Liu et al. reported rs10946398 of *CDKAL1* was independently associated with DR in Chinese Han population [100]. Complement 5 (*C5*) rs2269067 GG genotype and -2518 GG genotype, G allele of *MCP-1*, and rs1927914 of *TLR4* have conferred risks for proliferative DR of type 2 DM patients in Chinese Han population [101–103].

Long duration of diabetes, hyperglycemia, and hypertension are the most consistent risk factors for the progression of DR. From 50 to 90% of patients develop DR after more than 20 years of suffering diabetes. About 1% reduction in glycated hemoglobin (HbA1c) serum level is associated with an approximate 35% reduction in risk of DR development, 15–25% reduction in DR progression, 25% reduction in vision-threatening DR, and 15% reduction in blindness. A 10 mm Hg reduction in systolic blood pressure was reportedly associated with an approximate 40–50% reduction in DR progression [84]. Cataract surgery, pregnancy, puberty, and nephropathy are other common risk factors for the progression of DR.

17.4 Conclusive Remarks

Retina plays essential roles in vision. A dysfunctional retina leads to various retinal diseases, which could result in vision loss or even irreversible blindness. Recent advancements in genetic studies have identified retina genes. There are similarities and differences in the effects of genetic factors among different ethnic populations. In future, intensive studies in gene effects and environmental influences should be conducted for understanding the mechanisms and devising new treatment modes for various retinal diseases.

Compliance with Ethical Requirements Jingna He, Wai Kit Chu, Li Ma, Calvin C.P. Pang, and Guy L.J. Chen declare that they have no conflict of interest.

No human or animal studies were performed by the authors for this article.

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18 Leber Congenital Amaurosis in Asia

Sharola Dharmaraj, Anshuman Verma, P. Sundaresan, and Chitra Kannabiran

Abstract

Leber congenital amaurosis (LCA) is a heterogeneous infantile retinal dystrophy presenting with severe visual loss, nystagmus, sluggish pupillary responses and an extinguished electroretinogram (ERG). LCA accounts for 5% of inherited retinal degenerative disorders worldwide. To date at least 30 genes are known to either cause or be associated with this condition. The genes perform a structural or functional role in the visual pathway. Mutations in several of these genes causing LCA have been identified in Asian populations (*AIPL1*, *ALMS1*, *CABP4*, *CCT2*, *CEP290*, *CLUAP1*, *CRB1*, *CNGA3*, *CRX*, *CTNNA1*, *CYP4V2*, *GDF6*, *GUCY2D*, *IFT140*, *IMPDH1*, *IQCB1*, *KCNJ13*, *LCA5*, *LRAT*, *MERTK*, *MYO7A*, *NMNAT1*, *OTX2*, *PEX1*, *PNPLA6*, *POC1*, *PRPH2*, *RD3*, *RDH12*, *RPE65*, *RPGRIP1*, *SPATA7* and *TULP1*). An increased rate of consanguinity in some Asian populations has been noted, and gene identification using homozygosity mapping and testing for common mutations is possible, but the prevalence of mutations is not always identical to cohorts in the Western world. The advent of next-generation, whole genome and exome sequencing in addition to gene chip technology have revolutionised genetic and molecular diagnosis. Phenotype-genotype correlation of this disorder in some instances has made the choice of laboratory diagnosis rapid and easier. An accurate genetic diagnosis has become mandatory to access upcoming treatment options. Gene therapy for LCA has been encouraging recently as shown in the clinical trials involving *RPE65*-related LCA both in canines and humans.

Keywords

LCA · *RPE65* · Clinical trials · Gene therapy · Consanguinity · Mutations · Asian · Heterogeneity · Genotype · Phenotype · Treatment · Sequencing · *AIPL1* · *ALMS1* · *CABP4* · *CCT2* · *CEP290* · *CLUAP1* · *CRB1* · *CNGA3* · *CRX* · *CTNNA1* · *CYP4V2* · *GDF6* · *GUCY2D* · *IFT140* · *IMPDH1* · *IQCB1* · *KCNJ13* · *LCA5* · *LRAT* · *MERTK* · *MYO7A* · *NMNAT1* · *OTX2* · *PEX1* · *PNPLA6* · *POC1* · *PRPH2* · *RD3* · *RDH12* · *RPE65* · *RPGRIP1* · *SPATA7* · *TULP1*

S. Dharmaraj (✉)
Moorfields Eye Hospital, London, UK

A. Verma · P. Sundaresan
Aravind Medical Research Foundation, Aravind Eye Hospital, Madurai, India

C. Kannabiran
LV Prasad Eye Institute, Hyderabad, India

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18.1 Introduction

Leber congenital amaurosis is one of the most severe forms of retinal dystrophy presenting in infancy or early childhood with the absence of photoreceptor function [1]. LCA is a genetically and clinically heterogenous disorder causing profound visual loss, nystagmus, poorly reactive pupils and a markedly diminished electroretinogram (ERG) [1, 2]. Theodor Leber in 1869 first described the condition, but confirmation of the diagnosis was made easier by Adolphe Franceschetti, a Swiss ophthalmologist who noted the attenuated electroretinographic responses in patients with LCA in 1954. Theodor Leber even in those early days had noted its clinical heterogeneity. The heterogeneity of LCA had been well documented prior to the era of molecular diagnosis [3]. However, genetic heterogeneity was established only in the latter part of the last century. Visual acuity of <6/60 in the better eye is defined as blindness [4]. Currently at least 30 genes have been implicated in LCA, and they are involved in preserving photoreceptor and retinal pigment epithelial structure and function. The same genes implicated in LCA in some instances also cause other retinal disorders like retinitis pigmentosa, cone-rod dystrophy and early-onset retinal dystrophy. LCA is at the severe end of the genetic continuum of photoreceptor dysfunction and loss, while cone-rod dystrophy is at the milder end of this spectrum. In addition LCA may also be associated with non-retinal conditions. Mutations in genes causing LCA are linked with cerebellar disease (Joubert syndrome), epiphyseal disease (Saldino-Mainzer syndrome), renal disease (Senior-Løken syndrome) and neural tube defects (Meckel-Gruber syndrome).

18.2 Epidemiology

Hereditary retinal disease accounts for approximately 5% of blindness worldwide, and Leber congenital amaurosis accounts for 5% of all inherited retinal dystrophies [5]. LCA accounts for 1–3 in 100,000 live births per year [5]. The incidence of RP, the more common retinal dystrophy, is 1 in 3500 live births [6]. However, RP appears to be more common in regions with a high degree of consanguineous unions, for example, Saudi Arabia and South India [7]. Recessive gene defects may be higher in consanguineous populations. For example, in the south of India where maternal uncle marriages and first-cousin marriages still continue to the present day because of cultural, social or economic reasons, the occurrence of autosomal recessive disease is increased depending on the closeness of the genetic relationship. In inbred populations where individuals are related in more than one way, the risk of recessive genetic disease is further increased.

Retinal abnormalities, one of the major causes of childhood blindness, account for 22% of childhood blindness in India [4, 8].

The clinical history has been well documented over the last century, and LCA accounts for a proportion of blind school admissions each year in several countries in Asia. The incidence of retinal blindness in a referral hospital in Bengal was 9.5% [9]. Most cases of LCA are transmitted in an autosomal recessive fashion, but dominant forms of LCA have been described [10–12]. LCA like RP is also observed in conjunction with other systemic and syndromic disorders, like cerebellar disease (Joubert syndrome), epiphyseal disease (Saldino-Mainzer syndrome) and renal disease (Senior-Løken syndrome). Decreased visual acuity as a result of heritable retinal disease occurs in 1 in 3000 people [13]. More recently the genotype of many of these syndromic forms has been identified. Currently more than 261 retinal disease genes have been identified (RetNet URL https://sph.uth.edu/retnet/).

18.3 Clinical Diagnosis

The diagnosis of heritable retinal disease has been based on the clinical features. However, with the knowledge of molecular information and greater use of imaging and electroretinography, a more accurate diagnosis is possible.

Molecular genetics has brought better understanding to the inheritance of retinal dystrophies, the role of functional proteins and their biochemical pathways.

The diagnosis of heritable retinal disease is possible following the elucidation of a detailed family history and thorough clinical investigations. Examination includes not only the affected individuals but also potential carriers and other family members who may have subtle clinical findings. Family history and examination will elucidate the inheritance pattern. The clinical examination consists of assessing best corrected visual acuity, optical assessment (under cycloplegia in children) with slit-lamp biomicroscopy and dilated fundoscopy. Other relevant investigations include electrophysiological (ERG, dark adaptation curves and EOG) and psychophysical testing (static or kinetic visual field tests) and imaging and molecular studies. Imaging includes fundus photography, optical coherence tomography, scanning laser ophthalmoscopy, fundus fluorescein angiography and retinal autofluorescence. It is also important to perform a thorough medical and genetic examination in an effort to identify other clinical features suggestive of a syndromic form of retinal degeneration.

LCA includes both peripheral and central retinal disease leading to profound loss of vision early in life. In several inherited retinal disorders, photoreceptor cell death occurs as a result of apoptosis [14]. RP, the commonest retinal dystrophy, is characterised by progressive cell death of rods and cones. Cone cell loss occurs when rod outer segment degenerates to more than 75% as observed by electroretinography [15]. Cone loss correlates spatially and temporally with rod loss and this has been noted in pathological examination too [16, 17]. Retinal dystrophies could present in early infancy, whereby the arrested development of the photoreceptors is followed by degeneration. Early-onset severe retinal dystrophy has been defined as a subset of the LCA spectrum. When dystrophies present in later life, the degeneration occurs after the photoreceptors have developed, but their survival is not sustained. Loss of rod photoreceptors causes loss of peripheral vision, motion detection and decreased vision in scotopic conditions (nyctalopia). Night blindness and loss of peripheral visual fields are noted. Loss of cone photoreceptor cells results in loss of central vision (acuity loss), trichromatic colour discrimination (dyschromatopsia), vision loss in photopic conditions (hemeralopia) and ocular discomfort under photopic conditions (photoaversion or photophobia). In many retinal degenerations, apoptosis of the photoreceptor cells leads to decreased oxygen need. This altered oxygen tension stimulates autoregulation in the retinal vasculature causing attenuation of the blood vessels as a secondary effect noted ophthalmoscopically along with optic disc pallor.

Patients with LCA present in early infancy with wandering or roving eyes, photophobia and an inability to focus. Often there is a history of photophobia or photo attraction. Eye poking (digito-ocular phenomena of Franceschetti) is often noted in a large number of patients, often resulting in enophthalmos due to atrophy of orbital fat. The entoptic phenomena induced by eye poking may be a habitual behaviour observed in some patients. Digito-ocular signs noted in about 25% of patients include recurrent eye rubbing, eye pressing and eye poking and are not pathognomonic of LCA [18–20]. The presence of enophthalmos or eye poking did not show an adequate correlation with the molecular diagnosis. Sequelae of this habitual visual behaviour may include enophthalmos, keratoconus and ocular infections [21]. However, it is often very difficult to break this habit in children.

Decreased pupillary responses to light are observed in most patients. The retinae on ophthalmoscopy are either essentially normal in their appearance or show varying degrees of pigmentary changes [22, 23]. Additional ocular features may include cataracts, keratoconus and keratoglobus [23–25]. Keratoconus, observed in some patients, may be explained by genetic susceptibility or the secondary effect of the digito-ocular phenomena [23, 26, 27].

The overall phenotype in LCA is influenced by pathological changes in non-retinal tissues. The presence of cataracts, keratoconus and high

grades of refractive errors further increases the severity of the phenotype. High hypermetropia and myopia have also been reported [28]. Strabismus, too, has been a common finding and is often related to nystagmus and poor oculomotor control. To be able to minimise altered head position and nystagmus in some patients, surgical correction is undertaken.

Visual acuity in this disease ranges from light perception (LP) to vision 20/400, although rarely better visual acuity of 20/80 has been observed [23]. Relatively better vision has been noted in patients with *CRB1* and *RPE65* mutations [29]. Progression of the disorder is characterised by worsening visual function, increased retinal pigmentary and atrophic changes, attenuation of retinal vasculature and optic nerve atrophy. This was observed in one third of LCA patients for which the underlying gene defects were not identified [20]. Worsening is also noted in patients with other ocular findings like keratoconus and cataract, which add to the decreased clarity of the media [23, 26].

The electroretinogram (ERG) is non-detectable, extinguished or markedly reduced as a result of both rod and cone photoreceptor dysfunction. ERGs obtained in early infancy quite often need to be recorded again in 1 year for reliability.

Since an LCA-like phenotype may co-exist with a significant systemic disease, a measure of clinical suspicion must accompany each workup of an infant with LCA. Relevant investigations include ERG, serum very-long-chain fatty acid (VLCFA) assays, abdominal ultrasonography, MRI (magnetic resonance imaging) and developmental assessment. VLCFAs are raised in peroxisomal disease, which may mimic the retinal phenotype of LCA along with liver, spleen and central nervous system involvement. The presence of cystic changes in the kidneys or liver on ultrasonography is indicative of Senior-Løken or Arima syndrome, respectively. When renal disease is identified in Senior-Løken syndrome, morbidity increases; hence it is important to screen patients ultrasonographically and biochemically. Early renal transplants in these patients prolong survival. Constant monitoring of renal function is mandatory throughout childhood in these patients. Hand X-rays showing cone-shaped epiphysis may be useful in making the diagnosis of the Saldino-Mainzer disease. An MRI study of the brain is essential to detect abnormalities of the cerebellar vermis associated with Joubert syndrome and also to screen for other associated structural anomalies. A consultation by a neurogenetics team and a metabolic workup are important. The history of developmental milestones, visual behaviour, psychomotor delay and social behaviour are important in understanding development, as LCA has been associated with both autism and psychomotor delay. Psychomotor development is delayed in the setting of blindness due to the lack of adequate visual input which is essential for early development and neurobiological processing [18, 30]. Neuro- and psychomotor developmental delay was noted in 8.1% of patients with LCA [31]. Clinically, it is also important to elicit a detailed family history, draw a complete pedigree and chart ethnicity and genealogical origins. In the light of understanding the phenotype of LCA better, it is relevant to elicit the history of photophobia, photo attraction and night blindness. It is also important to ascertain milestones, regression and visual behaviour in terms of stability, progression or deterioration.

The differential diagnosis of LCA includes peroxisomal disorders (Zellweger disease, adrenoleukodystrophy and infantile Refsum disease), neuronal ceroid lipofuscinosis (NCL) and various forms of chorioretinal dystrophy (i.e. congenital stationary night blindness, achromatopsia, juvenile RP, albinism and high myopia). Albinism is the most common misdiagnosis [32]. Cortical blindness, delay in visual maturation and optic nerve hypoplasia may also be mistakenly diagnosed as LCA since all these disorders are associated with severe visual loss. In peroxisomal disease, retinal dysfunction is characterised by markedly attenuated photopic and scotopic ERGs and is associated with changes in the central nervous system. Peroxisomes are ubiquitous cell organelles involved in lipid metabolism. Peroxins

encoded by the PEX family of genes are responsible for various aspects of assembly and biogenesis of peroxisomes. Peroxisomes contain vitamin A storing lipid droplets that are distributed along the basal and lateral surfaces of the retinal pigment epithelium where receptors for retinal binding protein (RBP) are located. Műller cells also contain a high number of peroxisomes. The exact mechanism of loss of photoreceptor function is not completely understood. However, the phenotypic appearance of the retina along with the electroretinographic abnormalities is similar to LCA. The presence of photoreceptor dysfunction has been attributed to mutations in the PEX genes.

The presence of LCA with nephronophthisis in Senior-Løken syndrome; nephronophthisis, cone-shaped epiphyses of the hand and cerebellar ataxia in Saldino-Mainzer (conorenal) syndrome; and vermis hypoplasia, oculomotor anomalies and neonatal respiratory apnoea in Joubert syndrome has been documented [33, 34]. Joubert syndrome has been associated with LCA and multicystic kidneys [35]. Alström syndrome presents with LCA, cardiomyopathy, short stature, deafness and diabetes mellitus. Although cardiomyopathy is noted at infancy, diabetes mellitus occurs in the second or third decade [36]. The Arima syndrome, cerebro-oculo-hepatorenal syndrome, is a rare though well-defined entity that includes hepatic involvement. The clinical heterogeneity of this renal-retinal syndrome is indicated by the variable age of onset of the renal and retinal abnormalities. The link between the renal and retinal systems in Senior-Løken (renal-retinal) syndrome is the cilium. Homozygous mutations in *NPHP1* on chromosome 2q13, encoding nephrocystin and NPHP4 (nephroretinin) on chromosome 1p36 are one cause of the clinical syndrome of Senior-Løken. The interaction of nephrocystin, nephroretinin and the tubulin of cilia forms a complex in the primary cilia and co-localises in the renal epithelial cells. In the retina, the connecting cilium in photoreceptor cells plays a key role in the transport of molecules from the inner to the outer segment.

Renal dysplasia, pigmentary retinal dystrophy, cerebellar ataxia and skeletal dysplasia constitute syndrome Saldino-Mainzer or conorenal syndrome, named because of the cone-shaped epiphyses of the hands. The Saldino-Mainzer (conorenal) syndrome includes renal histopathologic changes consistent with a primarily glomerular disorder and short distal phalanges with cone-shaped epiphysis. Interstitial fibrosis and tubular cysts lead to renal insufficiency [37].

The cerebello-oculo-renal (CORS) syndromes include Joubert and other syndromes that exhibit a "molar tooth sign" on magnetic resonance imaging which consist of cerebellar aplasia or hypoplasia, thickened superior cerebellar peduncles and a large interpeduncular fossa [38]. Pathologically this represents significant brainstem malformation.

The LCA observed in patients with Joubert syndrome is stable, although symptoms associated with renal or hepatic involvement present in childhood or early adolescence, necessitating careful observation of blood pressure, blood urea nitrogen, urinalysis, liver function tests and abdominal ultrasonography to detect early renal and hepatic pathology. Visual impairment presents in early infancy. The presence of tachypnoea admixed with periods of central apnoea, seizures and brainstem abnormalities help make the diagnosis of Joubert syndrome. High-quality magnetic resonance imaging (MRI), polysomnograms and ophthalmic evaluation to assess visual function are indicated.

Mutations in the gene encoding the ciliary/centrosomal protein *CEP290* (NPHP6) or nephrocystin-6 lead to Joubert syndrome, and due to interactions with ATF4, a transcription factor involved in cAMP-dependent cyst formation, it is now possible to understand the phenotype of retinal degeneration and renal failure. *CEP290* is located in renal epithelial centrosomes and in the connecting cilia of photoreceptors [39]. Mutations in *CEP290* were found to be the most frequent cause of LCA in North American and West European populations [40].

18.4 Pathogenesis

Few pathological studies on retinal degenerative disease have been reported in which the genetic defect was known. Similarly, few studies on the morphological appearance (light and electron microscopy) of the retina in patients with LCA have been undertaken. Studies of prenatal LCA gene expression and regulation in the foetus will provide greater depth in understanding the cellular basis of this disorder. Case studies of *CRX, RPE65* and *GUCY2D* –related LCA have been reported.

CRX expression in the foetus is known to occur at 10–12 weeks in the foveal region when the rods are generated in the retina, and this is followed by further *CRX* expression that proceeds towards the peripheral retina [41].

An important comparative pathological study examined retinal tissue from a foetus with *RPE65* mutations and compared the histopathology and immunohistochemistry with an age-matched normal foetal retina [42]. Compared to normal foetal retina, the affected retina displayed cell loss and thinning of the outer nuclear (photoreceptor) layer and decreased immunoreactivity for key phototransduction proteins and aberrant synaptic and inner retinal organisation. The homozygous *RPE65* mutation C330Y abolished detectable expression of *RPE65* within the RPE of affected eyes, and ultrastructural examination revealed the presence of lipid and vesicular inclusions not seen in normal RPE. The mutant eye demonstrated thickening, detachment and collagen fibril disorganisation in the underlying Bruch's membrane, and the choroid was distended and abnormally vascularised, in comparison with the control eye [42]. These human foetal findings contrast with the late-onset ocular changes observed in animal models, indicating the problems of inferring human retinal pathophysiology from animal models [43]. This work also underlined the early prenatal pathogenic insult and congenital nature of human LCA in contrast to human RP, which is a disease of later onset.

In retinal tissue obtained from a 22-year-old patient with LCA harbouring a novel heterozygous *H82Y* mutation in *AIPL1*, histopathological examination showed almost total absence of photoreceptor cells, decreased ganglion cells, increased vacuolisation of the nerve fibre layer with retinal gliosis and a characteristic alteration of arterioles with venular dilation. Increased areas of retinal atrophy, sclerosis and attenuation of the blood vessels and pigment accumulation were also observed. Both the RPE and the Bruch's membrane were intact with a normal optic nerve [44]. This appearance correlates well with the phenotypic appearance observed in *AIPL1*-associated LCA [26]. Human retinal degeneration in both *GUCY2D*- and *RPE65*-associated LCA appears to occur prenatally, leading to profound photoreceptor dysfunction [45]. Interestingly, although the retinal appearance on ophthalmoscopy may appear normal, histopathological studies may show different changes as observed in the foetal appearance of both *GUCY2D*- and *RPE65*-associated LCA retinas with decreased photoreceptor cells and disorganised outer segments. This may have important implications in the considerations for clinical trials in the prenatal and postnatal stages and the extent of photoreceptor cell rescue possible. As more human foetal retinal tissue is studied, greater understanding of gene function in early development may be possible.

18.5 LCA Genetics

The main goal of research into the genetics of LCA is to identify the molecular basis of the disease and to develop therapies. LCA is largely inherited in an autosomal recessive pattern; autosomal dominant inheritance has been described infrequently. Being inherited in an autosomal recessive disorder, homozygosity (identity-by-descent (IBD) mapping has been a useful tool for identifying genes causing LCA, especially in families with consanguineous unions. However, more recently in the last decade, targeted whole exome sequencing and DNA microarrays have ushered in greater identification of novel genes and mutations. Almost a decade ago, mutations

Table 18.1 Causative LCA and LCA-associated genes

LCA type	Gene symbol	Protein	No. of exons	Chromosomal locus	Visual function	Disorders
LCA1	*GUCY2D*	Retinal guanylate cyclase 2D	20	7p13.1	Synthesis of cGMP during visual cycle	ARLCA
						CORD
LCA2	*RPE65*	Retinoid isomerohydrolase	14	1p31	11-cis-retinol regeneration from all-trans-retinyl ester in retinoid cycle process	AR LCA
						ESORD
LCA3	*SPATA7*	Spermatogenesis-associated 7	15	14q31.3	Not clear	LCA, Juvenile RP
LCA4	*AIPL1*	Aryl-hydrocarbon-interacting protein-like	6	17p13.1	Protein trafficking, folding and stabilization in phototransduction process	LCA, RP
LCA5	*LCA5*	Lebercilin	9	6q14.1	Protein transport across the photoreceptor connecting cilium	AR LCA
LCA6	*RPGRIP1*	Retinitis pigmentosa GTPase regulator-interacting protein 1	25	14q11	Protein transport processes into the photoreceptor outer segment	AR LCA
						AR LCA and neurodevelopmental delay
LCA7	*CRX*	Cone-rod homeobox	4	19q13.1	Transcriptional factor regulating photoreceptor cell-specific gene transcription	AD LCA
						AD cone-rod dystrophy, RP
LCA8	*CRB1*	Crumbs 1 cell polarity component	18	1q31-q32	Photoreceptor morphogenesis	AR LCA RP
LCA9	*NMNAT1*	Nicotinamide nucleotide adenylyltransferase1	9	1p36	NAD biosynthesis	AR LCA
						ESORD
						AR SLS and LCA
LCA10	*CEP290*	Centrosomal protein 290	60	12q21.32	Localization of ciliary and phototransduction proteins in retinal photoreceptor cells	AR LCA
						AR SLS
						AR Joubert syndrome
						AR Meckel syndrome
LCA11	*IMPDH1*	Inosine monophosphate dehydrogenase 1	18	7q31.3-q332	Retinal guanine synthesis	AD LCA AD RP
LCA12	*RD3*	Retinal degeneration 3	3	1q32.3	Outer segment ciliary transport of guanylate cyclase	AR LCA
LCA13	*RDH12*	Retinal dehydrogenase12	9	14q24.1	Reduction of cis and trans retinal in the retinoid cycle	AR LCA
						ARRP

(continued)

Table 18.1 (continued)

LCA type	Gene symbol	Protein	No. of exons	Chromosomal locus	Visual function	Disorders
LCA14	*LRAT*	Lecithin retinol acyltransferase	5	4q31	Chemical transformations of vitamin A (all-trans-retinol) into 11-cis retinal, in retinoid cycle	LCA, early-onset ERSOD
LCA15	*TULP1*	Tubby-like protein 1	15	6p21.3	Protein transport across the photoreceptor connecting cilium	AR LCA
						AR ESORD
LCA16	*KCNJ13*	Potassium voltage-gated channel subfamily J member 13	3	2q37.1	Regulating potassium channel across cell membrane	Snowflake degeneration
LCA17	*GDF6*	Growth differentiation factor 6	2	8q22.1	Development of the eye	AD microphthalmia, Klippel-Feil syndrome
						Chorioretinal coloboma
						(LCA-like)
LCA18	*PRPH2*	Peripherin 2	5	6p21.1	Stabilization of the outer segment of the photoreceptors	Digenic RP, ADRP, progressive macular degeneration, macular dystrophy
						LCA
…	*OTX2*	Orthodenticle homeobox 2	5	14q22.3	Differentiation of photoreceptors	Microphthalmia (MCOPS5) and combined pituitary hormone deficiency (CPHD6)
						LCA-like
…	*IFT140*	Intraflagellar transport140	40	16p13.3	Ciliary transport between the inner and outer segment of photoreceptors	AR Saldino-Mainzer syndrome
						ARRP
						ARLCA
…	*PNPLA6*	Patatin-like phospholipase domain-containing protein 6	37	19p13.2	Membrane lipid homeostasis	Oliver-MacFarlane syndrome, trichomegaly-retina pigmentary degeneration and dwarfism
…	*CABP4*	Calcium-binding protein 4	8	11q13.1	Maintenance of synaptic function	Stationary night blindness type 2B
						Cone-rod synaptic disorder
	POC1	POC1 centriolar protein B	13	12q21.33	Ciliogenesis basal body and centrosome integrity	AR JS, PKD and LCA AR CORD
…	*MERTK*	MER tyrosine kinase proto-oncogene	24	2q13	Outer segment phagocytosis by RPE	ARRP ARRCD
						EOSORD
						ARLCA

…	*IQCB1*	IQ motif-containing protein B1 (nephronophthisis 5)	16	3q13.33	Photoreceptor ciliary function	AR SLS
						LCA
…	*ALMS1*	Centrosome and basal body-associated protein	23	2p13.1	Photoreceptor ciliogenesis Centriole structure and function	ARLCA
						Alström disease
…	*CNGA3*	Cyclic nucleotide-gated channel alpha 3	9	2q11.2	Visual and olfactory signal transduction	AR achromatopsia, ARCORD
…	*MYO7A*	Myosin 7A	55	11q13.5	Photoreceptor organization	Usher syndrome (1B), deafness AR, decreased vestibular function, retinal degeneration,
						USH3-like
	CTNNA1	Catenin alpha 1	27	5q31.2	Cell adhesion	Butterfly-shaped pigment dystrophy ADMD
						LCA
	CCT2	Chaperone-containing TCP-1	17		Chaperone stability	AR LCA
	DTHD1	Death domain containing 1	12	4p14	Signalling and apoptosis	Myopathy and LCA
	CLAUP1	Claustrin-associated protein 1	15	16p13.3	Essential for ciliogenesis and photoreceptor maintenance	LCA
	PEX1	Peroxisomal biogenesis factor 1		7q21.2	Biogenesis of peroxisomes	Infantile Refsum, Zellweger, neonatal adrenoleukodystrophy
						(LCA-like retinal appearance)
	CYP4V2					ARLCA
						Bietti's corneoretinal dystrophy

RPE Retinal pigment epithelium

in all LCA- associated genes accounted for 70% of the clinically diagnosed LCA in Caucasian populations [46]. LCA occurs due to mutations in the genes involved directly or indirectly in the visual pathways. There are at least 30 such genes identified, and new genes are constantly being discovered (Table 18.1). With discovery of genes causing LCA comes the understanding of protein function. Photoreceptor structure and morphogenesis involve *CRB1*, *CRX*, *GDF6* and *OTX2*, while phototransduction involves *AIPL1*, *GUCY2D* and *RD3*. *LRAT*, *RDH12* and *RPE65* are involved in the retinoid cycle. Interestingly the detection of genes involved in the ciliome has increased remarkably in the last decade, and these include *ALMS1*, *CEP290*, *CLUAP1*, *IFT140*, *LCA5*, *RPGRIP1* and *TULP1*. Mutations in these genes cause ciliopathies.

These known genes account for disease in 70–80% or more of patients with LCA currently, suggesting that new undiscovered genes account for the remaining 20–30% cases. A recent study from India using a 20-LCA gene panel involving 92 patients with LCA detected mutations in 61% [47]. LCA is a rare disease, and many studies undertaken worldwide have not always mentioned the origin of the patients, and LCA cohorts reported in the West include DNA from the wider diaspora of Asian patients from the East, too. The number of LCA-causing mutations detected has increased over the past two decades, and most studies have not always included all the most recently identified genes in their sequencing, and as a result the relative percentages of the mutational load are quite variable as are the populations studied. Nevertheless due to enormous international collaborative efforts, significant understanding of both the clinical phenotype and the genotype has been possible. A large number (~800) of mutations are known to cause LCA (mutation database), but many of these are private mutations found only in 1 family. The mutations have been missenses, deletions, insertions and frameshift and splice site mutations. Missense mutations account for the greatest number of mutations in any cohort. Several mutations cause null alleles and truncated proteins leading to loss of function. *CEP290*, *GUCY2D*, *CRB1*, *RPE65* and *CRX* account for a major portion of LCA. The individual mutational frequency of these genes may vary from one ethnic population to another [46, 48, 49]. *CEP290* mutation is relatively frequent in European and American patients with LCA while remarkably low or absent in other regions, including India. [50]. A particular *CEP290* mutation, c.2991 + 1655A > G that causes truncated protein formation due to aberrant splicing, accounts for 20% of LCA in Europe [40]. Recently, genes (*ALMS1*, *CNGA3*, *IQCB1* and *MYO7A*) related to syndromic or non-syndromic retinal disorders have been reported to be genetically linked with LCA [51]. Interestingly, genes causing LCA, like *CRX*, *LRAT*, *MERTK*, *RPE65*, *CEP290* and *TULP1*, have also been implicated in other early-onset retinal degeneration like retinitis pigmentosa (RP) and cone-rod dystrophy (CORD). Both RP and CORD are less severe than LCA, and the onset of disease is not as early as LCA.

For their study of LCA, the ocular genetic laboratories in LV Prasad employed direct sequencing of candidate genes for mutations that are disease-associated. The patients recruited were from different regions of India. Testing of a few selected candidate genes for LCA – *GUCY2D*, *CRB1*, *RD3*, *RPE6* and *NMNAT1* – showed a combined frequency for all these genes of approximately 12% in a total of 100 probands tested. Among these, pathogenic changes were detected in *GUCY2D* (2%), *CRB1* (2%), *RPE65* (4%), *RD3* (<1%) and *NMNAT1* (4%). Mutations in the *RD3* gene are associated with <1% of cases with LCA from different parts of India and in different populations across the world reported in the literature with a similar low frequency of mutations [52, 53].

The frequency of mutations in different populations is remarkable. While in Chinese and Indian families the commonest gene mutations are in *GUCY2D,* in Saudi Arabia *TULP1* mutations are most common. In Belgian populations *CEP290* was most commonly mutated, while in Indonesian, Italian and Danish cohorts, RPE65 mutations were the commonest [47, 48, 54–57].

The above tables have listed many genes associated with LCA; however, several of them cause an LCA-like fundus appearance which could be normal or show varying degrees of vascular attenuation or pigmentary disturbance. Genes like *GDF6*, *CABP4*, *PEX* and *ALMS1* cause other disorders.

MYO7A PNPLA6, *CYP4AV2*, *CLAUP1*, *DTHDI*, *CCT2*, *CTNNA1* and *CNGA3* need more research to validate them as genes causative of LCA, and there are likely others that require more molecular studies to validate them as genes causing LCA.

Using homozygosity mapping and linkage analysis, several genes causing LCA were identified. Whole exome sequencing and candidate analysis contributed vastly to the identification of more causative genes for LCA. The genes associated with transduction include *GUCY2D*, *RD3* and *AIPL1*, while those involved with morphogenesis are *CRB1*, *CRX* and *OTX2*. Retinoid recycling in the RPE are dependent on *RDH12*, *LRAT* and *RPE65*. The ciliome is important for maintaining structure and function in the photoreceptor cell and is involved in transport of metabolites and impulses between the inner and outer segment of rods and cones. *ALMS1*, *CEP290*, *CLAUP1*, *IFT140*, *IQCB1*, *LCA5*, *RPGRIP1*, *SPATA7* and *TULP1* play a pivotal role in the ciliome.

18.5.1 *AIPL1*

The aryl-hydrocarbon-interacting protein-like (*AIPL1*) gene located on chromosome 17p13.1 has six exons and is expressed in the inner segment, nucleus, perinuclear region, synaptic terminals and cytoplasm of the photoreceptor cells. It is expressed in the developing retina and is essential for the normal development of the rods and cones [75–77]. Mutations in *AIPL1* cause AD CORD, juvenile ARRP and ARLCA [76]. In LCA, *W278X*, the commonest mutation in *AIPL1*, alters the structure, stability and transport of the protein [78]. In keeping with greater loss of rod function both in heterozygote carriers of *AIPL1*-associated LCA and in patients with LCA with mutations in both *AIPL1* copies, the survival of adult rods is more dependent on *AIPL1* function than cones. However, the absence of *AIPL1* in early retinal development leads to both rod and cone dysfunction as *AIPL1* is required for the normal development of both rod and cone photoreceptors [79]. The phenotype is severe, presenting early with loss of vision and varying areas of atrophy and hyperpigmentary changes in the retina. Keratoconus has been described in some probands [26, 47]. The outer retina in young patients with *AIPL1* mutations is preserved, and it has been observed on OCT, providing an opportunity for gene therapy [80]. Mutations in this gene causing LCA have been observed in Indian, Pakistani, Indonesian, Arab and Chinese populations (Table 18.2).

18.5.2 *ALMS1*

ALMS1, a centrosome and basal body associated protein, is involved in photoreceptor ciliogenesis. In infancy the presence of markedly decreased visual function, marked by severely extinguished rod and cone function coupled with mutations in *ALMS1*, identified the phenotype in these patients as Alström disease [81]. Alström disease is associated with obesity, loss of visual function, hyperopia, non-recordable ERGs and hearing loss. Cardiomyopathy, hyperinsulinemia, hypertension and hypertriglyceridemia may be associated, too. Mutations causing LCA were identified in Saudi Arabian patients [82].

18.5.3 *CCT2*

CCT2 is a chaperone-containing T-complex polypeptide, and mutations involved chaperone instability. Patients with LCA have a severe ocular phenotype with attenuation of retinal vasculature, disc pallor and thin disorganised retina. Eye poking was observed [83]. Two novel compound heterozygous mutations in a Chinese family have been reported c.1198A > G and c.1547G > A.

Table 18.2 Mutation and Asian LCA

No.	Gene OMIM	*Gene*	% Reported in worldwide populations	Asian LCA
1	600179	*GUCY2D*	6–21	Japanese (Hosona et al.)
				Saudi Arabian (Safieh et al., Li et al.)
				Bedouin Saudi Arabian (Gradstein et al.)
				Indian (Verma et al., Srilekha et al., Srikrupa et al.)
				Chinese (Chen Y et al., Li Y et al.)
2	180069	*RPE65*	3–16	Japanese (Wada et al., Katagiri et al.)
				Saudi Arabian (Li Y et al.)
				Indian (Ramprasad et al., Verma et al., Srilekha et al., Srikrupa et al.)
				Arab (Beryozkin et al.)
				Indonesia (Sitorus et al.)
				Chinese (Li Y et al.)
3	609868	*SPATA7*	–	Indian (Srilekha et al., Srikrupa et al.)
				Arab (Beryozkin et al.)
				Chinese (Li Y et al.)
4	604392	*AIPL1*	5–10	Indian (Verma et al., Srilekha et al., Srikrupa et al.)
				Arab (Beryozkin et al.)
				Indonesia (Sitorus et al.)
				Chinese (Li Y et al.)
				Pakistani (Khaliq et al., Damji et al.)
5	611408	*LCA5*	1–2	Indian (Ramprasad et al., Mackay et al., Srikrupa et al.)
				Pakistani (Ahamad et al., Mackay et al.)
				Afghan (Mackay et al.)
				Iraqi (Mackay et al.)
				Taiwanese (Mackay et al.)
				Korean (Seong et al.)
				Chinese (Li Y et al., Mackay et al.)
6	605446	*RPGRIP1*	4–6	Pakistan (McKubbin et al.)
				Saudi Arabian (McKubbin et al., Khan et al.)
				Egyptian (Abouzeid et al.)
				Korean (Seong et al.)
				Chinese (Li Y et al., Huang et al., Chen Y et al.)
				Indian (Srikrupa et al.)
				Japanese (Suzuki T et al.)
				Thai (Jinda et al.)
				Iranian (Imani et al.)
7	602225	*CRX*	1–3	Chinese (Chen Y et al., Li Y et al.)
				Indian (Verma et al., Srikrupa et al.)
				Japanese (Nakamura et al.)

(continued)

Table 18.2 (continued)

No.	Gene OMIM	*Gene*	% Reported in worldwide populations	Asian LCA
8	604210	*CRB1*	9–13	Palestinian (Abouzeid et al., Beryozkina et al.)
				Israeli (Beryozkina et al.)
				Indian (Srilekha et al., Srikrupa et al.)
				Saudi Arabian (Li et al.)
				Iranian (Ghofrani et al.)
				Chinese (Chen Y et al., Yang et al., Li Y et al.)
				Korean (Seong et al.)
				Pakistan (Khaliq et al.)
				Japanese (Kuniyoshi et al.)
9	608700	*NMNAT1*	–	Chinese (Jn X et al.)
				Han Chinese (Tong H et al.)
				Pakistan (Koenekoop et al.)
				Indian (Srikrupa et al.)
				Japanese (Coppeiters et al.)
				Thai (Jinda et al.)
10	610142	*CEP290*	20	Arab (Aboussair et al.)
				Chinese (Chen et al.)
				Chinese (Li et al.)
				Thai (Jinda et al.)
11	146690	*IMPDH1*	8	Chinese (Chen et al.)
				Japanese (Wang et al.)
12	180040	*RD3*	<1	Indian (Srikrupa et al.)
13	608830	*RDH12*	4–5	Indian (Sunderamurthy et al., Srilekha et al., Srikrupa et al.)
				Chinese (Li et al.)
				Japanese (Kuniyoshi et al.)
14	604863	*LRAT*	<1	(Srikrupa et al.)
15	602280	*TULP1*	1	Arab Israeli (Abbesi et al.)
				Saudi Arabian (Li et al.)
				Arab (Beryozkin et al.)
				Chinese (Chen et al.)
				Indian (Srikrupa et al.)
16	603208	*KCNJ13*	–	Middle Eastern (Sergouniotis et al.)
				Jordian (Pattnaik et al.)
				Indian (Srikrupa et al.)
17	601147	*GDF6*	–	
18	60813	*PRPH2*	–	
19	610125	*OTX2*	–	
20	614260	*IFT140*	–	Chinese (Xu et al.)
21	603197	*PNPLA6*	–	
22	608965	*CABP4*	–	
23	614784	*POC1*	–	Iraqi (Beck et al.)
24	604705	*MERTK*	2	Chinese (Li et al.)

(continued)

Table 18.2 (continued)

No.	Gene OMIM	*Gene*	% Reported in worldwide populations	Asian LCA
25	609237	*IQCB1*	3	Turkish (Otto)
				Indian (Verma et al., Srilekha et al., Srikrupa et al.)
				Chinese (Chen et al.)
				Saudi Arabian (Wang et al.)
				Thai (Jinda et al.)
26	606844	*ALMS1*	–	Chinese (Li et al.)
				Saudi Arabian (Safieh et al.)
				Thai (Jinda et al.)
27	600053	*CNGA3*	–	
28	276900	*MYO7A*	–	
29	608970	*CTNNA1*	–	Thai (Jinda et al.)
30	605139	*CCT2*	–	Chinese (Minegish et al.)
31	616979	*DTHD1*		
32	616787	*CLAUP1*		
33	602859	*PEX1*		
34	608614	*CYP4V2*		Thai (Jinda et al.)

Table references: Hosono et al. [58], Verma et al.[59], Srilekha et al. [60], Chen et al. [61], Gradstein et al. [62], Li et al. [55, 63], Katagiri et al. [64], Wada and Tamai [65], McKay et al. [66] McKibbin et al. [67], Suzuki et al. [68], Nakamura et al. [69], Beryozkin et al. [70] Kuniyoshi et al. [71], Imani et al. [72], Huang et al. [73], Li et al. [74]
OMIM Online Mendelian inheritance in man, *AD* Autosomal dominant, *AR* Autosomal recessive, *CORD* Cone-rod dystrophy, *LCA* Leber congenital amaurosis, *RP* Retinitis pigmentosa, *SLS* Senior-Løken syndrome, *JS* Joubert syndrome

18.5.4 *CEP290*

CEP290 is a centrosomal protein (290 kDa) that controls ciliary transport between the inner and outer segments of photoreceptors. *CEP290* was the first gene noted to cause syndromic LCA [40]. The commonest intronic mutation (c.2991 + 1655A > G) creates a strong splice donor site and inserts a cryptic exon in the CEP290 mRNA (Jacobson SG IOVS 2017 [40, 84]. OCT and full-field sensitivity testing showed dissociation of structure and function of the photoreceptors. *CEP290* mutations are the commonest mutations in Western populations and are a frequent cause of non-syndromic LCA [85]. Mutations cause diffuse retinal degeneration. Foveal cones are preserved as noted on OCT [86]. Clinical features include renal failure, ataxia, hypotonia and Joubert syndrome [85]. Mutations were reported in Meckel-Gruber syndrome [87]. *CEP 290* mutations were noted in patients with anosmia [88].

18.5.5 *CLUAP1*

CLUAP1 (clusterin-associated protein 1) is associated with ciliogenesis and is critical in photoreceptor function. Hypomorphic mutations were identified in *CLUAP1* (hypomorphic mutations result in partial function). Compound heterozygous mutations have been detected in Joubert syndrome and orofacial with digital overlap syndrome, too [89]. Hypomorphic mutations associated with LCA have been identified in this gene [90].

18.5.6 *CRB1*

The crumbs homolog 1 (CRB1) gene was isolated by subtractive hybridisation. It is expressed in the apical membranes of retinal photoreceptors [91]. *CRB1* maps to 1q31–32.1 and consists of 12 exons. It is expressed in the adult and foetal brain. CRB1 is involved in cell-cell interaction and may be responsible for the maintenance of retinal cell

polarity. *CRB1* is essential for the integrity of the external limiting membrane and photoreceptor cell morphogenesis [92]. It is needed to maintain an organised layer of photoreceptor cells during light exposure, and in its absence adhesion between the photoreceptor cells and the Möller cells is lost resulting in both structural and functional changes. Light exposure results in an increase in focal retinal lesions [93]. Mutations in this gene are associated with a severe form of RP, RP12, and with LCA [94–97]. Mutations in *CRB1* were detected in patients with RP and Coats-like vasculopathy and in patients with RP and preserved para-arteriolar retinal pigment epithelium [98]. The retinal phenotype is variable and nummular pigment clumping and white dots have been observed in LCA. Slow progression of the disease has been noted [91, 95, 97]. Mutations also result in RP without para-arteriolar RPE preservation. High hyperopia was noted with a compound heterozygous mutation in a consanguineous family of Palestinian descent [99]. Characteristically an unlaminated thickened retina is noted on OCT [100].

18.5.7 *CRX*

The cone-rod homeobox gene (*CRX*) on chromosome 19q13 encodes a transcription factor important in embryonic photoreceptor development. *CRX* belongs to the otx/otd homeobox family of genes and is similar to the human OTX1 and OTX2 homeodomain proteins [101]. It is expressed in the pineal gland and in developing and mature retinal photoreceptor cells. *CRX* is also expressed in the inner nuclear layer [41]. It is necessary for the maintenance of normal cone and rod function [102]. This cone-rod otx-like photoreceptor homeobox transcription factor is capable of trans-activating several photoreceptor cell-specific genes and plays a crucial role in differentiation. Mutations in *CRX* are known to cause AD LCA, autosomal dominant cone-rod dystrophy and autosomal dominant retinitis pigmentosa [11, 12, 103, 104]. Some AD mutations in CRX are associated with LCA [10, 11, 105]. Interestingly CRX interacts with NRL and missense mutations in NRL cause ADRP and not LCA, while recessive mutations cause thickening of the retina and an unusual pigmentary retinopathy [106, 107]. Patients have nonprogressive markedly decreased visual acuity with diffuse pigmentary retinal changes and ateliotic maculas [108, 109].

18.5.8 *CNGA3*

Cyclic nucleotide-gated cation channel (*CNGA3*) encodes one of the alpha subunits of the ion channels necessary for phototransduction. Mutations have been detected in patients with AR achromatopsia. Mutations associated with LCA were detected in a Saudi Arabian pedigree [51].

18.5.9 *CTNNA1*

CTNNA1, catenin alpha 1, plays a role in cell adhesion and connects cadherins on the plasma membrane to the actin filaments inside the cell. Mutations cause AD butterfly-shaped macular dystrophy [110]. Mutations in a Thai patient with LCA were reported [111].

18.5.10 *CYP4V2*

Cytochrome P450 family 4 subfamily V is a gene that encodes a member of the cytochrome P450 protein involved in oxidizing substrates in the metabolism of fatty acid precursors. Mutations have been noted in Bietti corneoretinal crystalline dystrophy and more recently in LCA [111, 112].

18.5.11 *DTHDI*

DTHDI (death domain containing 1) is involved in cell signalling and apoptosis, and mutations have been reported in a patient with LCA and myopathy [113].

18.5.12 *GDF6*

Growth differentiation 6 encodes a secreted ligand of the TGF-beta superfamily of proteins. This protein is required for bones and joints in the axial skeleton. Mutations cause Klippel-Feil syndrome, dominant microphthalmia, chorioretinal coloboma and a phenotype not similar to LCA, although an LCA reference has been assigned to this gene.

18.5.13 *GUCY2D*

Retinal guanylate cyclase 2D, the first gene linked to LCA on chromosome 17p13.1, encodes a retinal protein that is involved in phototransduction. *GUCY2D* synthesises the intracellular messenger of photoreceptor excitation cGMP and is regulated by the intracellular calcium-sensitive proteins GCAPs. *RETGC1* is a protein involved in photorecovery in the phototransduction cascade, and mutations in the gene hinder the restoration of cGMP in the rods and cones to the basal state, resulting in permanent closure of the cGMP-gated channels [114]. Mutations in *GUCY2D* were first identified in patients with LCA [114]. However, mutations in patients with CORD have been detected [115]. Nevertheless more mutations causing LCA in cohorts worldwide have been noted [47, 116, 117]. It is one of the major causes of LCA, and of the 140 mutations reported, 88% cause AR LCA.

The fundus in infants appears normal initially, but in later years varying degrees of peripheral pigmentary changes are noted. A greyish tapetal reflex has been noted in many of these patients. However, the ERG has been vastly diminished, and the OCT shows perifoveal thinning [117, 118].

18.5.14 *IFT140*

IFT140 encodes an intraflagellar transport complex A (IFTA) that pays a role in the maintenance of transport in the connecting cilia between the inner and outer segments of photoreceptors. Mutations cause a ciliopathy involving the skeleton and eye. *IFT140*-associated LCA causes poor vision, nystagmus, peripheral RPE mottling with depigmented spots in the retina and non-recordable ERGs. The phenotype consists of retinal pigment epithelial atrophy [119].

18.5.15 *IMPDH1*

Inosine monophosphate dehydrogenase 1 (*IMPDH1*) on chromosome 7q31.3–q32 is involved in cyclic nucleoside metabolism within photoreceptors. Mutations in *IMPDH1* cause ADRP (RP10) and ADLCA [120, 121].

18.5.16 *IQCB1*

The IQ calmodulin-binding motif-containing B1 protein encodes nephrocystin 5 that localises to the primary cilia of renal epithelium and connecting cilium of photoreceptors. The *IQCB1* protein interacts with RPGR and calmodulin in the photoreceptor connecting cilia. The LCA phenotype includes distinct retinal blood vessel straightening and altered pigmentation around the vascular arcades [122]. Senior-Løken syndrome involves cystic kidney disease (nephronophthisis) and retinitis pigmentosa or Leber congenital amaurosis; homozygous mutations were detected in several patients with LCA patients, some of whom later developed kidney disease [123]. In addition to LCA, psychomotor delay and midface hypoplasia were observed in an Arab proband who had a mutation in the IQCB1 gene. Sequence variants were noted in the *ACAT1* and *FGFR2* genes, too [51].

18.5.17 *KCNJ13*

KCNJ13 (potassium channel) that controls inward potassium flux in RPE is expressed in the apical microvilli. A novel mutation resulting in loss of channel function was identified in a Jordanian pedigree. Early progressive retinal

degeneration involving both rods and cones was noted [124]. Retinal degeneration and thinning, with nummular pigment distribution, have been observed [125]. Mutations cause autosomal dominant snowflake vitreoretinal degeneration [126]. Vitreoretinal dystrophy and cataracts were noted in Saudi Arabian families [127].

18.5.18 *LCA5*

LCA5 (protein product, lebercilin) maps to chromosome 6q14.1, a region rich with retinal genes. The gene *LCA5* was identified as it was part of the ciliome, and mutations in the *LCA5* gene account for about 2% of LCA [128]. The affected individuals of a family (Old Order River Brethren) of German-Swiss descent mapping to chromosome 6q11–q16, presented with visual acuities in the order of 20/100–20/400, high hyperopia and normal fundi in early infancy and childhood and mutations, were identified in lebercilin. Interestingly one of the patients showed a heterozygous change in GUCY2D (C2174T) which did not cosegregate with the disease and may exert a modifying influence on the phenotype causing an increase in retinal degeneration [118, 129]. However, the phenotype of another family from Pakistan mapping to the same locus was quite different. Progressive, severe maculopathy consisting of pigmentary disturbance and retinal pigmentary atrophy was noted [130]. Atrophic retinal maculopathy was a distinct feature in the Pakistani kindred; however, despite a similar ophthalmoscopic appearance, the *LCA5* locus was excluded in a Turkish pedigree [131]. Mutations in *LCA5* also cause cone dystrophy wherein the phenotype showed no remarkable peripheral retinal changes, and novel biallelic mutations were detected in a Chinese sibship [132]. The LCA phenotype is severe, presenting with nyctalopia, hyperopia and markedly decreased visual function. Hypopigmented white spots have been noted on fundoscopy [133]. Retinal pigment atrophy and loss of photoreceptors in the outer retinal layer are noted on OCT, and fundus autofluorescence shows a hypofluorescent macula and a hyperfluoroscent fovea signifying continued metabolic activity of the foveal cones. Most mutations have been null mutations and have been identified in several Asian probands [67]. Homozygous mutation was noted in a Pakistani family who presented with LCA and cataract [134]. *LCA5* mutations were identified in several Asian populations [135].

18.5.19 *LRAT*

Lecithin retinol acyltransferase (*LRAT*) maps to 4q31 and encodes a polypeptide of 230 amino acids. *LRAT* is synthesised in the retinal pigment epithelium [136]. *LRAT* catalyses the initial series of reactions in the conversion of all trans-retinol to all trans-retinyl ester in the RPE and plays a vital role in the regeneration of the visual chromophore. Patients with mutations in *LRAT* causing LCA present with decreased visual function, night blindness and photophilia, and severe retinal atrophy and fibrosis have been noted on fundoscopy. OCT confirmed photoreceptor loss and disrupted retinal lamination was noted [137]. Mutations in *LRAT* lead to early-onset severe retinal dystrophy [138].

18.5.20 *MERTK*

The MER tyrosine kinase proto-oncogene is expressed in RPE and is involved in phagocytosis. Mutations are known to cause ARRP, childhood-onset rod-cone dystrophy and LCA. Mutations have been noted in Chinese families with LCA [63].

18.5.21 *MYO7A*

MYO7A Myosin 7A encodes a myosin, a mechanochemical protein when mutated causes Usher syndrome IB characterised by deafness, abnormal gait and retinal degeneration [139]. LCA was noted in a Saudi Arabian pedigree [51].

18.5.22 *NMNAT1*

Nicotinamide nucleotide adenylyltransferase 1 on chromosome 1p36 is involved in nicotinamide adenine dinucleotide biosynthesis and localises to the cell nucleus. *NMNAT1* mutations reduce the enzymatic activity of *NMNAT1* in NAD biosynthesis which affects protein binding and neuroprotection. The locus was linked initially to a Pakistani pedigree and mutations were identified later [140]. Visual acuity was the perception of light in all affected individuals. Posterior subcapsular lens opacities, macular staphylomas, varying degrees of retinal white spots and pigmentary retinopathy with optic atrophy were noted [141].

The LCA phenotype is one with severe macular and optic atrophy with attenuation of retinal vasculature and marked visual loss. The *E257K* mutation was the commonest and was observed in a great majority of patients in several studies [142–144].

18.5.23 *OTX2*

Orthodenticle homeobox 2 (*OTX2*) is a transcription factor involved in the differentiation of photoreceptors and interacts with CRX, NRL and TR-beta 2. Mutations in addition to early-onset retinal dystrophy also cause short stature as a result of abnormal pituitary function. Fundoscopy shows fine hyperpigmentation of the RPE and the peripapillary zone [145].

18.5.24 *PNPLA6*

Patatin-like phospholipase domain containing protein 6 (*PNPLA6*) is involved in the deacetylation of membrane lipids in the central nervous system. This protein is expressed in the inner segment of photoreceptors. In one case report of a compound heterozygous mutation in *PNPLA6*, the patient had a reduction of vision with nystagmus. It causes severe retinal degeneration and diminished ERGs [146]. Patients with Oliver-McFarlane syndrome (OMS) have a complex phenotype characterised by blindness due to severe photoreceptor degeneration, dwarfism due to pituitary growth hormone deficiency, trichomegaly and progressive alopecia [147]. Occasionally, OMS is associated with mental retardation, distal muscle weakness/wasting and ataxia, due to axonal peripheral neuropathy. Mutations have been identified in patients with spastic paraplegia [148]. Human organophosphorous-induced delayed neuropathy (OPIDN) occurs when *PNPLA6* becomes phosphorylated by these organophosphates, followed by dealkylation of the phosphoryl enzyme, inhibiting the catalytic domain.

18.5.25 *POC1*

Centriolar protein chlamydomonas homolog 1b (POC1) localises to the centriole close to the cilium of photoreceptors and plays a role in maintenance and duplication of the centriole in the basal body of cells. It also localises to the synapsis in the outer plexiform layer of the retina. Mutations were first detected in patients with CORD [149]. Mutations causing CORD were reported in a Turkish cohort [150]. LCA with polycystic kidney disease and Joubert syndrome was reported in an Iraqi family of consanguineous origin [151].

18.5.26 *PRPH2*

Peripherin 2 retinal degeneration slow (*PRPH2*) helps stabilise the outer segment of photoreceptors. Mutations are known to cause ADRP, autosomal dominant macular dystrophy, retinitis punctata albescens, digenic RP and AR LCA. The children stare at lights. Asymptomatic parents (heterozygous carriers) show peripheral retinal pigmentary changes [152]. Recessive *PRPH2* mutations cause prominent maculopathy in adulthood.

18.5.27 *RD3*

Retinal degeneration 3 (*RD3*) is an accessory chaperone protein for transport of GUCY2D and GUCY2F from photoreceptors. Mutations cause macular and retinal degeneration [52, 53]. Mutations cause early-onset severe retinal dystrophy. Phenotypically they present with disorganisation of the retina with thinning of the inner nuclear, ganglion cell and nerve fibre layers [153].

18.5.28 *RDH12*

RDH12 (LCA13) localises to the inner segment of the photoreceptors and is involved in the transformation of 11-cis retinol to 11-cis retinal of the phototransduction cascade. The phenotype presents with marked retinal degeneration and macular atrophy [154].

18.5.29 *RPE65*

The retinal pigment epithelium 65 (*RPE65*) gene on chromosome 1p31 encodes a protein which plays a pivotal role in retinoid metabolism. *RPE65* is a non-glycosylated 65 kD microsomal protein expressed in retinal pigment epithelium and is conserved in mammals, reptiles and birds. It is necessary for the production of 11-cis vitamin A. Mutations in *RPE65* disrupt the synthesis of the opsin chromophore ligand 11-cis retinal and are partial to total loss of isomerization [155]. The deactivation of sensory transduction by opsins causes light-independent retinal degeneration in LCA. *RPE65* defects result in progressive photoreceptor cell death. Both LCA and severe ARRP have been reported with mutations in *RPE65* [56, 116, 156–162]. The most number of RPE-65 mutations have been detected in Denmark [54]. Chorioretinal atrophy and pigmentary retinopathy have been noted in patients [163]. Relatively preserved central vision and retinal structure was noted in Chinese patients [164].

18.5.30 *RPGRIP1*

The retinitis pigmentosa guanosine triphosphatase (GTPase) regulator-interacting protein 1 gene (*RPGRIP1*) on chromosome 14q11 encodes a protein that is involved in disc morphogenesis through regulating actin cytoskeleton dynamics [165]. RPGRIP1 consists of 24 exons, and the predicted protein consists of 1259 amino acids. *RPGRIP1* was identified as an interactor of RPGR (retinitis pigmentosa GTPase regulator); both localise to the connecting cilium. *RPGRIP1* anchors RPGR to the connecting cilium. Mutations in *RPGR* cause X-Linked RP (RP3) [166]. Mutations in *RPGRIP1* lead to LCA and AR CORD [167–169]. Increased frequency of mutations were noted in patients from Pakistan and Saudi Arabia. [67].

In an Egyptian proband, neurodevelopmental delay and LCA were noted with a severe ocular phenotype and marked decrease on VEP testing [170]. The phenotype is severe and presents with poor vision and drusen-like deposits [163]. Bone spicule-like pigmentary changes have been noted in the retina [47].

18.5.31 *RDH12*

The retinol dehydrogenase 12 (*RDH12*) gene on chromosome 14q23.3–q24.1 has been implicated in early-onset severe retinal dystrophy and LCA [171, 172]. It encodes a photoreceptor protein that is important in retinoid metabolism. The gene consists of 7 exons and encodes a protein of 316 amino acids. *RDH12* belongs to the superfamily of short-chain alcohol dehydrogenases and reductases. *RDH12* may be involved in the conversion of 11-cis retinol to 11-cis retinal during the regeneration of cone visual pigments. *RDH12* is expressed predominantly in the neuroretina in photoreceptors [173]. Phenotype includes atrophic macular lesions and disorganised retinal architecture [47, 154].

18.5.32 *SPATA7*

LCA3/SPATA7 spermatogenesis-associated protein 7 localises to the cilium of the photoreceptors and is involved in protein trafficking and recruiting RPGRIP to the cilium. Mutations cause peripheral retinal degeneration and juvenile RP. Phenotype comprises severe progressive rod-cone degeneration and relatively preserved foveal structure [174]. Patients presenting with this subset of LCA had enophthalmos, nystagmus in early life, moderate hyperopia, variable strabismus, profound visual loss, retinal pigment mottling and clumping. Older individuals of the Saudi Arabian kindred had perception of light, nuclear or cortical cataracts, optic atrophy, clumping of retinal pigment, atrophic maculas and vascular attenuation [47, 175].

18.5.33 *TULP1*

Tubby-like protein (*TULP1*) gene is analogous to the tub gene in mice that is responsible for maturity-onset obesity, insulin resistance, retinal degeneration and neurosensory hearing loss. Tub is known to cause early progressive retinal degeneration in mice. Human *TULP1* maps to chromosome 6p21.3, consists of 15 exons and is expressed exclusively in the retina. Mutations in this gene are known to cause early retinal dystrophy [176]. The human tubby TU and tubby-like proteins *TULP1*, *TULP2* and *TULP3* belong to a highly evolutionarily conserved gene family and play an important role in obesity, sensorineuronal degeneration and development [177]. The TULPs are expressed in the brain, retina and cochlea. *TULP1* localises to the perinucleolar cap region in photoreceptor cells and is involved in protein trafficking. *Tulp1* labelling is noted in the inner segments of rods and cones in foetal retinas at ~8 weeks [178]. *TULP1* is a transcription factor involved in the control of downstream genes in photoreceptors and is involved in retinal phagocytosis. Mutation screening and direct sequencing revealed a novel splice site mutation in an Arab Israeli family [179]. Mutations causing LCA were noted in an Indian cohort [47].

Although some genes have been associated with LCA, it has not always been very appropriate. Better phenotyping and genotyping with more extensive laboratory work could eliminate this problem. *Capb4* is a gene mutated in cone-rod synaptic disorder and not LCA as reported. Genes associated with colobomas and photoreceptor dysfunction in addition to central nervous system abnormalities are not generally causative of LCA.

18.6 Molecular Testing

Clinicians managing patients with LCA now can send a simple cheek swab or blood to genetic labs. High-throughput screening methods such as LCA mutation chips and next-generation sequencing-based exome-sequencing could be undertaken to arrive at a genotype. DNA microarray-based chip (Asper chip, www.asper-bio.com) offers a rapid, cost-effective and accurate genotyping method for detection of all known disease-associated variations in LCA [180]. It contains a collection of specific probes which target specific variations. This chip provides a cost-effective option for mutation detection and may be used for molecular diagnosis to study LCA cohorts. Causal genes in LCA have been identified using linkage and homozygosity mapping, candidate gene analysis, whole genome and whole exome sequencing.

18.7 Genotype-Phenotype Correlation

LCA constitutes the most severe form of retinal dystrophy since retinal function is profoundly decreased, as evidenced by extinguished ERGs in infancy. Although progress in the understanding of the molecular mechanisms underlying LCA has steadily grown, genotype-phenotype correlations are not straightforward. However, the clinical heterogeneity of this disorder spares some individuals from extreme severity, allowing for some residual retinal function [116, 118, 161, 181–183].

The diagnosis and distinction between early-onset retinitis pigmentosa and LCA come with the extinguished or markedly attenuated ERG obtained in patients with LCA during early infancy. Leber in his early description of the disease included the early-onset severe retinal dystrophy. The phenotype in LCA is not only determined by the primary defect but is also influenced by the genetic background and the environment. The dysfunctional biochemical, structural and physiological pathways involved in vision in LCA have an impact on the phenotype. All the gene products involved in visual function are diverse in their subcellular locations and functions. The timing of the insult, too, influences the phenotypic appearance. The natural passage of time further alters the retinal appearance in LCA. Mutations in the known genes can be detected in about 70% of patients with LCA [184]. Early severe onset retinal dystrophy is noted more commonly in patients with mutations in *LRAT*, *RDH12* and *RPE65*. A severe LCA phenotype with markedly decreased visual function is noted in patients with mutations in *AIPL1* and *GUCYD*. Both LCA and ESORD share remarkable clinical and genetic overlap.

The natural history of LCA has been reported to be stable; however, slow deterioration, or in a few cases some transient improvement, too, has been noted [23, 116, 183, 185–187]. Summarising several longitudinal studies, stability of clinical course and visual function was observed in 75% of patients with LCA, deterioration in 15% and an improvement in visual function in 10% of patients [185, 186, 189]. Comparing the phenotypes in patients with a molecular diagnosis, patients with *GUCY2D* mutations have a stable clinical course, while those with *RPE65* mutations show progressive deterioration [162, 187, 190]. The collation of several studies with known genotypes reveals that more than half of the the majority of patients with LCA run a stable visual course, while approximately a fourth of them improve and less than a fourth deteriorate.

Maculopathy has been noted in patients with mutations in *TULP1 CRB1 AIPL1 NMNAT1*. Twenty-five genes and their phenotypes were analysed by [191]. Mutations common in Western populations include *CEP290 CRB1, GUCY2D RDH12* and *RPE65*, while common mutations in Asian populations include *TULP1* and *GUCY2D*.

Mutations in the known LCA-associated genes may show overlapping phenotypes [29, 116, 117, 191, 192]. *CRB1* mutations have been detected in RP with PPRPE and also in A*RRP* without PPRPE, besides causing LCA [193]. As the number of patients in whom both genotypic and phenotypic data become available, it may be possible to generate a clearer picture regarding significant genotype-phenotype correlations.

All LCA-related genes show a high degree of allelic heterogeneity and this contributes to the increased phenotypic variability [194]. The phenotypic variability seen in patients from diverse populations with mutations in the same gene further proves the diversity of this disease.

RDH12-associated LCA presents as progressive rod-cone degeneration perhaps due to the longer survival of cones in these retinas as is also observed in *RPE65*-associated LCA. Interestingly, both these genes are involved in retinoid metabolism. With the increasing inv olvement of rods in LCA and the longer survival of cones, photopic function may be preserved longer, as noted in *RDH12, AIPL1 and RPE65*-associated LCA. Early rod dysfunction is also noted in the carriers of *AIPL1*-associated LCA [26]. Peripapillary RPE sparing was noted in patients with *RDH12* mutations. Retinal thinning was noted on spectral-domain OCT. Generalised fundus autofluorescence was diminished [195].

RPE65 and *TULP1*-associated LCA present as severe early-onset retinal dystrophies. Absence of autofluorescence is observed in the *RPE65*-associated phenotype [182]. Photoreceptor function that can be rescued as delineated by the presence of normal autofluorescence in some patients with LCA in the second decade of life has been reported [196]. Increasingly poor vision at night is a common complaint in patients with mutations in genes affecting the retinoid metabolism as noted in *RPE65* mutations [116, 187]. Patients with mutations in *AIPL1* and *RPE65* have a prominent pigmentary maculopathy [26].

AIPL1-associated LCA shows early macular involvement in a significant proportion of patients.

Patients with *CRB1* mutations in several instances show a Coats-like phenotype and a thickening of the retinal layers by OCT, unlike that seen in other patients. Retinal thinning is easier to understand as it occurs due to thinning of the RPE and the photoreceptor layer. Both the presences of a Coats-like phenotype and RP with PPRPE due to *CRB1* mutations hitherto lack a sufficient explanation. GUCY2D-associated LCA is a severe but stable phenotype with frequent complaints of photophobia, due perhaps to the impaired production of cGMP with the persistent closure of the cGMP-gated cation channels [114]. CRX- and IMPDH1-associated LCA is the only subtype of LCA with a dominant mode of transmission, wherein affected parents have affected offspring and several de novo mutations have been identified [11, 12, 121, 186].

Mutations in rare recessive diseases such as LCA may have a founder basis. Therefore, the presence of founder mutations was explored. A common Finnish founder mutation in *GUCY2D* causing LCA (2943delG) has been detected even in an outbred population. F565S appears to be a common mutation in GUCY2D occurring in families of North African descent [197]. Another common founder mutation detected in RPE65 (Y368H) accounts for disease in an isolated Dutch population [162]). A founder mutation (c.95-2A > T; IVS2-2A > T) in *RPE65*, in a North African Jewish community, has made it possible to enrol these patients for gene therapy [198]. To date, few recurrent mutations have been found in the LCA genes. The intronic *CEP290* mutation (c.2991 + 1655A > G) which creates a cryptic splice donor site resulting in the insertion of an aberrant pseudoexon into more than 50% of the *CEP290* transcripts and the *W278X* mutation in *AIPL1* are common. The latter has been detected homozygously in consanguineous families as well as in combination with other mutations in patients born of non-consanguineous union, and it shows a high incidence in populations of South East Asian descent [26, 199]. The presence of this mutation in most instances leads to a very severe LCA phenotype [26]. Though most LCA variants are rare, some are recurrent, and hence, it is worthwhile to use a microarray disease chip as a first-pass screening tool in every new case of LCA. The use of the LCA disease chip in any given patient with the clinical diagnosis of LCA has more than 50% likelihood of detecting one or two mutations [184].

The frequency of mutations in each subtype of LCA is dependent on the populations screened. In an Indonesian LCA cohort, *RPE65* mutations were predominant (9.5%), followed by *AIPL1* mutations (4.8%), while no mutations in *GUCY2D* were detected [56]. On the other hand, in a largely North African population, *GUCY2D* mutations were more common [117, 200]. A predominance of *CRB1* mutations (~13%) was observed in the Netherlands and Germany, while *CRB1* mutations accounted for 11% and 10% of patients from the United States and North Africa/France, respectively [97, 98, 117]. A predominance of *GUCY2D* mutations has been detected in patients from Mediterranean countries [117, 200]. Patients of Asian descent have mutations in *GUCY2D*, *AIPL1* or *RPE65* [26, 47, 157]. Modifier genes studied so far play a small role in disease. In LCA the role of modifiers has been noted in disease progression. Ateliotic maculas (failure to achieve perfection) have been noted wherein the macula failed to develop or function as normal in CRX-related LCA [11, 12, 201, 202]. Severe ateliosis of the macula has been noted in *AIPL1*- and *RDH12*-related LCA, while foveal aplasia is noted in *NMNAT1*-related LCA.

Ocular coherence tomography has been a helpful aid in understanding the relative preservation of the retina and could potentially influence the nature of therapeutic intervention. Characteristically an unlaminated thickened retina is noted on OCT in *CRB1*-related LCA [100]. Normal foveal thickness with a central island of outer nuclear layer has been noted in *CEP290*, *LCA5*, *RPGIP1* and *TULP1*-related LCA on OCT [86]. *GUCY2D*-related LCA shows normal retinal thickness and a subnormal macular thickness. *CEP290* severe phenotype, some foveal cones were preserved as noted on OCT [86]. Peripapillary RPE sparing was noted in patients

with *RDH12* mutations, and retinal thinning was noted on spectral-domain OCT.

18.8 Carriers of LCA Mutations

It has been noticed that several phenotypically normal carrier parents harbouring heterozygous mutations in the LCA-associated genes have abnormalities in rod or/and cone ERGs, suggestive of mild rod or/and cone dysfunction [26, 31, 203]. Drusen-like deposits were noted in carriers of mutations in *RPE65*, *AIPL1*, *CRB1* and *RPGRIP1*, while mild peripheral chorioretinal atrophy was detected in carriers of *AIPL1* and *RPE65* mutations [31]. Retinal changes in the form of inferior retinal atrophy have been detected in carriers of *CRB1* mutations. These findings indicate that carriers of LCA-associated mutations can now be identified in several LCA families based on ERGs showing mild rod or cone dysfunction. Biallelic or triallelic mutations have been detected, and they may modify the phenotype [47].

18.9 Asian Perspective

Asian populations are greater than the populations in Western countries; however, published data from Asia regarding LCA is small. This disorder which affects 1–3 in a 100,000 worldwide is likely underreported in some parts of Asia. In regions where consanguinity is high, the prevalence may be higher. In some regions consanguinity is the norm and not an exception, making autosomal recessive disease more prevalent.

Sequencing Han Chinese and Korean genomes enabled identification of SNPs, variants and mutations in these ethnic groups. More Asian genomes may be sequenced in future which will aid in the better understanding of SNPs, variants and mutations in Eastern populations.

Just as some causal genes are greater in some Western populations (*CEP290* mutations), some may be greater in the diverse Eastern populations. Mutations in *CEP290* have not been reported in patients from Korea [204]. Studies from the Middle East, Korea, Japan, Indonesia, Pakistan and India have helped define mutations in LCA. *RPGRIP1* mutations are more common in the reported LCA cohort in Northern Pakistan [67]. TULP1 mutations were more common in Saudi Arabian pedigrees [55]. The detection of novel genes in Asian populations may be easier with homozygosity mapping especially in regions where consanguinity is high. In Asia, charting the genetic patterns and identifying the genotypes will also not only help identify phenotypes in the Asian populations but also in the Asian diaspora. It is also important to identify the genotypes and phenotypes of patients with LCA to prepare for therapy. Treatable forms of LCA in Asian populations may be more rapidly identified as both genetic and clinical diagnoses become more streamlined. Greater collaboration, reliable core sequencing facilities, sharing of clinical and genetic information and easy access to public databases have made it easier to collate data and understand the significance of various genetic changes. Genetic testing, as part of the clinical workup of ophthalmic patients, is more common now in several centres, and liaising with labs in different parts of the world to process samples of DNA and provide genotyping results will become more valuable to patients where complete facilities for all genetic diagnosis are not yet available. It has also become cost-effective to share facilities and use genetic laboratory services far from home to obtain useful information for patients and clinicians.

18.10 Management

The diagnosis for most LCA patients has meant a lifetime of coping with debilitating visual loss as there have been no treatment options. In the last few decades, greater molecular understanding of the disease has been possible, paving the way for a new look at treatment options. In a continuing effort to understand the evolution and natural history of the disease, it is important to examine the patients at regular periodic intervals. Serial fundus

photography, optical coherence tomography (OCT) and electroretinography (ERG) document any structural and functional changes.

For AR LCA, there is a one in four risks for offspring being affected when parents are carriers. Recurrence risk for the progeny of affected patients is low in the absence of consanguinity, as noted in a previous study [117]. In some parts of Asia, patients with this largely recessive disease may have a strong family history because of consanguinity. When considering AD LCA in genetic counselling, the recurrence risk for children of affected patients is 50%. The stigma of this blinding disease is worse outside the close confines of kinsmanship in some rural populations.

The present practical management for LCA patients is visual rehabilitation and training with visual aids. Supportive management has included the correction of refractive errors, cataract extraction, intraocular lens implantation and strabismus surgery. On the other hand, novel treatment for LCA is becoming a real option now, although considerable preparation is necessary in terms of genotyping and phenotypic analysis. Gene therapy trials for *RPE65*-associated LCA have been underway in four centres. Gene therapy involves a normal copy of a curative gene being introduced into target cells to restore its function. The success story of gene therapy for *RPE65*-associated LCA provides an insight into potential cures for other types of inherited childhood blindness.

Navigational vision and an ability to appreciate contrast are vital for patients. Rods, being necessary for cone survival, become essential to replace degenerating rods to salvage the cones and thus preserve vision. On the other hand, it may also be beneficial to protect the cones from rod-mediated cell loss. Either trophic factors or pharmacologically derived nano-molecules mimicking the same function could be used to prevent the degeneration of cones which are so vital for vision [17, 205, 206]. Gene therapy to correct the underlying molecular defect, pharmacotherapy, retinal cell transplantation and the use of visual prosthesis are the way forward.

18.11 Gene Therapy

As apoptosis may be the final common pathway of cell death in retinal degeneration, modifying the process could delay degeneration. Gene therapy using trophic growth factors enhances the survival both ultrastructurally and functionally of photoreceptors in retinal degeneration.

Great progress has been made in gene therapy studies and the use of animal models in retinal disease [207]. The retina is considered to be a region of immune privilege, thus making it a viable area for gene therapy [208]. Genetically based therapy could involve either replacement or suppression of dominant-negative mutations. Adenovirus vectors are non-enveloped, double-stranded DNA viruses capable of gene transfer in retinal cells. Adeno-associated and recombinant adenovirus vectors have been used successfully for gene transfer in the retina [209, 210]. Adeno-associated virus (AAV) and lentivirus have been used to transfect RPE cells with therapeutic genes to delay photoreceptor death in rodents [207, 211]. The size of genetic material to be packaged in viral DNA for effective transfection has led to the use of lentiviruses in animal models.

Intraocular gene transfer via subretinal injections, notwithstanding uveitis, has shown remarkable recovery of photoreceptor function in Briard dogs with *RPE65*-associated LCA. Swedish Briard dogs with a naturally occurring 4 bp deletion in *RPE65* showed improvement of electroretinographic function following injections with recombinant adeno-associated virus (AAV) carrying normal *RPE65* complementary DNA (cDNA) [212]. An added milestone in the treatment of congenital blindness as observed in LCA is the use of successful in utero gene therapy. The in utero delivery of human *RPE65* cDNA to the RPE cells using AAV2/AAV1 capsid in *RPE65* knockout mice results in efficient transduction with measurable levels of rhodopsin and restoration of visual function [213].

Gene replacement therapy for RPE-based retinal dystrophies rescues photoreceptor function as noted in the RCS rats with wild-type mertk

therapy and in Briard dogs with *RPE65* gene therapy [214]. Interestingly, out of 55 blind dogs used in the study, 90% began to recover vision which demonstrated that inherited blindness could be improved. This led to three contemporaneous human clinical trials in three different centres, including Moorfields Eye Hospital, UK; Children's Hospital of Philadelphia, USA; and Scheie Eye Institute of the University of Pennsylvania with Shands Children's Hospital of the University of Florida. Published results from these trials were ground-breaking [215, 216]. All the patients had a modest improvement in measures of retinal function and on subjective tests of visual acuity. These studies have provided the basis for further gene therapy trials in patients with LCA. Since then, several LCA gene therapy clinical trials accessing safety and efficacy have been performed successfully [217–219]. The results of clinical trials using *RPE65* gene therapy in adults initially and children later have proved the safety and efficacy of this form of therapy albeit with a non-persistence of visual restoration.

A crucial step in gene therapy for LCA involves the delivery of the vector carrying the *RPE65* gene that enables it to transfect the target cells to induce subsequent in vivo generation of the *RPE65* protein. The subretinal space has been used by many researchers to inject the vector as it acts as a relatively immune privileged site and also easily transfects the target cells – the retinal pigment epithelium and photoreceptors. In post-treatment follow-up, it was observed that safety and efficacy of gene transfer with rAAV2-*RPE65* vector in LCA patients extended to at least 1 year [220]. In the 3-year follow-up study, it was shown that there was no evidence of systemic dissemination of vector sequences and no evidence of humoral immune response or cell-mediated immune responses to AAV2 capsid or *RPE65* protein [221].

Posttrial objective measures included evaluation of the pupillary light reflex, nystagmus testing and optical coherence tomography. Subjective measures included standard tests of visual acuity, the Goldmann visual field examination and mobility testing to assess differences in the ability of the patients to navigate a standardised obstacle course before and after the therapy. These tests results have shown improvement in visual acuity, mobility, visual behaviour and retinal functions after treatment [222].

No serious adverse effects or systemic toxicity has been reported. However, some adverse outcomes that were observed in few cases include retinal detachment, choroidal effusions, transient ocular hypotony and ocular hypertension related with the administration of topical steroids. The outcome of a recent study for a long-term post-treatment follow-up in LCA patients as well as in canine models showed that gene therapy improves vision but photoreceptor degeneration progresses unabated [220]. The early effect of therapy up to 6–12 months sustains visual improvement, but over a period of time, there was no consistent improvement in visual acuity, and there was no improvement in the ERG along with the decrease in retinal thickness measured by OCT [223]. The lack of long-term effects of gene augmentation therapy suggests a need for a combinatorial strategy for effective outcome. Besides gene replacement therapy, more recently genetic editing has become an option for patients with the common Cep290 splice site mutation (c.2991 + 1655A > G) wherein antisense oligonucleotide (ASO) treatment restores pre-mRNA splicing thereby increasing CEP290 protein so necessary for efficient ciliary transport in photoreceptors [224, 225]. Clinical trials are now underway using ASO for Cep290-related LCA.

The *RPE65* gene replacement trials by Bainbridge et al. noted that the effect of the gene function decreases over time and the natural course of retinal degeneration continues. McGuire and Testa noted a sustained stable improvement of acuity nystagmus and visual filed over 3 years [215, 222, 226]. This gene replacement trial could also be applicable in LCA caused by mutations in *AIPL1 CEP290 GUCY2D LCA5 and RPGRIP* [227].

18.12 Pharmacotherapy

Human ciliary neurotrophic factor (CNTF) sequestered in semipermeable capsules and implanted in the vitreous cavity maintain cone survival [228, 229]. Since both rod and cone dysfunction occurs in LCA, the use of trophic factors in this disorder could be explored. The protective effect of neurotrophic factors like ciliary neurotrophic factor has been exploited in gene transfer, and a delay in retinal degeneration has been noted [230].

Oral treatment using multiple doses of QLT091001 (QLT) which is a stable synthetic compound converted in the body to 9-cis retinal that combines with opsin to form isorhodopsin and is able to activate the phototransduction cascade on exposure to light improves visual function in *RPE65 and LRAT*-related LCA [231]. This drug bypasses the block in the retinoid cycle and concentrates in the retina improving visual fields, acuity and functional MRI values.

18.13 Cell Transplantation

Replacement stem cells provide mutation-independent regenerative therapy. Since LCA is a heterogeneous disease implicating genes involved in several different pathways, the use of cell transplantation circumvents individual gene-related mechanisms of overcoming retinal degeneration. Transplantation serves to replace damaged cells or provide growth and survival factors to cells to prevent further cell death. The role of stem or precursor cell transplants in retinal degenerative disease provides a potential route to photoreceptor cell renewal. Photoreceptor survival following stem cell transplants occurs due to the release of trophic factors both close to the transplant and away from it [17, 206]. Rod-dependent cone viability factors play a role in the rescue process [232]. Human embryonic-derived stem cell RPE and pluripotent stem cell-derived RPE have been used in LCA to recover visual function [233]. Converting patient-specific somatic cells to be reprogrammed into pluripotent stem cells (iPSC) and fulfilling the need for functional RPE and photoreceptor cells are a step towards ameliorating immunologic adverse events noted in allogenic transplants [234]. iPSC cells have been used to evaluate novel mutation pathogenicity and gene-based rescue. Ciliogenesis has been restored using *CEP290* gene transfer in cells proving another avenue for gene- and cell-based therapy [235].

18.14 Retinal Prosthesis

Using the intact inner retina to carry impulses to the visual cortex has been possible using retinal prosthesis. The use of a visual prosthesis whereby electrical stimulation bypasses damaged nerve endings has been used in the treatment of retinal degenerative disorders [236]. Both the crude appreciation of the sensation of light and the ability to detect motion have been possible using these prostheses [237]. The Argus system works by direct stimulation of the inner retina via epiretinal microelectrodes. Visual information gathered by a video camera mounted on external glasses is converted to pixelated images by an external processor, and this is transmitted to the microarray electrode array at the macula which in turn transmits it to the occipital cortex via the optic nerve [238, 239]. The occipital cortex is not totally dysfunctional in the absence of light-sensitive input, but activation of the visual cortex is observed by positron emission tomography (PET) scanning and during functional magnetic resonance imaging while Braille reading. The period of cross-modal plasticity extends into the second decade of life in blind patients [240]. Transretinal electrical stimulation using multichannel arrays have shown results [241]. However, electronic photoreceptor prosthesis are limited by their biocompatibility, longevity, stability and image quality [242]. Neuroprosthesis have been used to help with visual rehabilitation [243]. Promising results with persistent VEPs were observed in canine models with an Okayama University-type retinal prosthesis (OURePTM),

a photoelectric dye-coupled polyethylene film that generates electric potential in nearby neurons in response to light [244].

Implantation of a cortical stimulation unit attached to a camera and a laptop computer has been able to increase visual potential [245]. As both tactile and auditory pathways are well developed in subjects with profound loss of vision, voice and tactile technology are being made use of to simulate vision [246]. Using optogenetics to restore degenerated retina relying on the intact inner neural pathway to convey impulses has offered some treatment possibility [247].

The genetic introduction of light-sensitive opsins to drive the visual pathway has been suggested [248].

18.15 Future Perspectives

Animal models of LCA have increased our comprehension of the disease and have provided an avenue for clinical trials, although they may not truly represent human disease. Molecular diagnosis in LCA will permit better prediction of visual prognosis.

Gene therapy, cell transplantation, pharmacogenomics-based therapy and retinal prosthesis are all future considerations in the treatment of this disease. Pioneering treatment for LCA involves issues of consent especially in the paediatric age group, ethical approval, safety and the efficacy of new clinical trials. With greater collaboration within the scientific community, the pharmaceutical industry and the regulatory bodies will propel the therapeutic options for this rare congenital retinal dystrophy. Providing support to patients and their families is furthered by support groups who have also helped accelerate research and involvement in clinical trials.

The aim of the 1000 Genomes Project was to find genetic variants with frequencies of at least 1% in the populations studied. The 1000 Genomes Project took advantage of cost-effective sequencing and was the first project to sequence the genomes of a large number of people, to provide a comprehensive resource on human genetic variation. The data from the 1000 Genomes Project was made available to the worldwide scientific community through freely accessible public databases.

Later the 100,000 Genomes Project, a UK Government initiative, was launched to sequence whole genomes from patients using the National Health Service. The focus is on rare diseases, cancers and infectious diseases. The genomic and clinical data are linked and made available to patients, clinicians and researchers making it possible to understand individuals and their diseases better when planning treatment options.

LCA in most instances results in the profound loss of photoreceptor function, and gene therapy directed at this disease involves long-term stable expression of transgenes for several decades. Although the pace of translational research in retinal degenerative diseases has been fairly fast, several difficulties still need to be overcome. The duration of gene expression over a lifetime to confer therapeutic efficacy, indelibility of translational control of transgene expression and the improved safety of therapy continue to be important issues.

New patient cohorts in Asia, in addition to characterised European/American cohorts, could be instrumental in finding new candidate genes LCA. In India established ocular research centres and medical research foundations are involved with research in patients with LCA focusing on involved genes, mutations, phenotypes and molecular mechanisms in the pathogenesis of the disease. Similar centres in other parts of Asia have been instrumental in identifying mutations in patients with LCA in order to stratify patients for treatment. Autozygosity-guided sequencing approach may be used as a powerful tool in detecting novel genes.

Finding novel genes expressed in the RPE and the photoreceptors using the Affymetrix GeneChip technology will provide valuable information regarding candidate genes, and this will be of great value in gene identification. Variability in the human genome in people of Asian origin is now being studied, and the

100,000 genome project (publically funded UK National Health Service project) will shed further light on the significance of variants in different ethnic groups and their impact on disease.

The first Asian genome of the Han Chinese and the later sequencing of the Korean genome have showed several differences in minor allele frequency, novel single-nucleotide polymorphisms (SNPs) and copy number variants (CNVs) which could potentially affect the expression of several genes. These insights have led to the importance of sequencing several other ethnic genomes in the 1000 genome project TGP. Cataloguing human variation in different populations will also be helpful in understanding minor allele frequencies, disease variants and traits in other Asian populations [249].

Despite targeted next-generation sequencing and analysis of copy number variants (CNV), causative mutations have not always been identified because they occur in non-coding regions and 5' UTR in disease genes. Including these hidden mutations in gene chips and panels will increase detection rates.

Understanding modifiers and their role in disease will shed light on the molecular mechanisms involved in LCA. Gene therapy in animal models of *AIPL1*, *GUCY2D*, *RPGRIP1* and *CEP290* has also been promising, paving the way for human trials. The establishment of better diagnostics and treatment options for LCA would herald a new phase of genomic medicine and hope for families.

Next-generation sequencing allows unparralled sequencing at an affordable cost in a timely fashion. Exome sequencing of the known LCA-related genes is a reliable first-pass test when the clinical diagnosis has been confirmed but the genotype is yet to be identified. The detection of founder mutations will simplify mutation detection in inbred populations. Outbred populations will validate the results obtained from inbred ones.

WES is a powerful tool for rapid analysis of known disease genes in large patient cohorts.

Most mutations detected are in coding regions of genes, but there is mounting evidence for hidden non-coding changes or structural variants altering protein function. The databases of eye diseases, exome variant server and exome sequencing data in addition of sharing of phenotypic information will increase the knowledge of the disease and provide valuable insight into treatment options that may be more gene-specific.

Autozygosity mapping being more time-consuming and less cost-effective in comparison with exome sequencing yields better rates of detection for pathogenic changes.

Providing replacement for the mutant factors, inhibiting apoptotic pathways or stimulating anti-apoptotic pathways, downregulating mutant gene expression, providing gene replacement and specifically targeting gene correction will help pave the way for treating this hitherto untreatable disease.

18.16 Summary

During the past two decades, several populations of patients have been tested genetically using gene panels; the numbers of genes incorporated in these panels increased year on year as more were discovered. The percentage of gene mutations identified has not always reflected the true frequency of gene mutations in any given population. However, certain mutations like the W278X in *AIPL1* are consistently higher in Asian populations. The frequency of the intronic mutation (c.2991 + 1655A > G) in *CEP290* is higher in outbred Western populations of America and Europe unlike in Asia. *CEP290* mutations have not yet been identified in Arabian populations. With the identification of more mutations in Asian cohorts, the LCA microarray mutation chip could be updated, and its use as a first-pass genetic screening tool could potentially yield greater results.

In the 1990s linkage analysis and candidate gene identification were employed in gene discovery. However, after the publication of the human genome, techniques such as next-generation sequencing (NGS), whole gene sequencing (WGS) and exome sequencing (WES) have enabled identification of greater numbers of LCA-related genes and causative mutations.

LCA, though described in the 1860s, more than a century and a half later our clinical understanding has improved. The genetic nature of this rare retinal disorder has offered greater insight and treatment options are now on the horizon. Identifying the LCA genotype in any given new patient has a higher success rate, as better and faster methods for gene identification at a lower cost are now possible. Gene therapy, in addition to pharmacotherapy and the use of electronic prosthesis, has made management of LCA conceivable finally translating research from the bench to the bedside.

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Compliance with Ethical Requirements All authors declare that he/she has no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients for being included in the study.

No animal or human studies were carried out by the authors for this article.

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Online Resources

Exome Variant Server
Human Mutation database
Exome Aggregation Consortium
ClinVar Databases.
https://clinicaltrials.gov/
Leiden online variation database
https://databases.lovd.nl/shared/transcripts/RPE65

19 The Genetics of Inherited Retinal Diseases in the Israeli and Palestinian Populations: A Lesson from Populations with High Rates of Consanguinity

Mor Hanany and Dror Sharon

Abstract

Inherited retinal diseases (IRDs) are disorders that cause visual loss mainly due to photoreceptor degeneration. The prevalence of IRDs in the Israeli and Palestinian populations was reported to be higher compared to other studied populations. The structures of the Israeli and Palestinian populations are unique mainly because of the large number of ethnic groups. In addition, high rates of consanguinity and intra-community marriages resulted in a high proportion of families with autosomal recessive inheritance patterns. The study of Israeli and Palestinian IRD families resulted so far in the identification of mutations in 74 IRD genes, including 23 novel genes that were identified mainly using the homozygosity mapping and whole exome sequencing techniques. The history and tradition of these populations led to common founder mutations that are usually subpopulation-specific. Such mutations allow a more efficient genetic analysis in searching for the causative gene. However, some founder mutations are shared among different ethnicities and are likely to be the result of a common origin of these ethnic groups, which may have an estimated divergence time of a few thousand years. There is a large variability of retinal phenotypes among patients, while mutations in the same gene can result either in the same phenotype or variable phenotypes that are usually mutation-dependent. There is currently no cure for the vast majority of IRD types; however recent advances bring new hope for curing or at least delaying the degeneration process in the near future.

Keywords

Consanguinity · Gene · Genotype-phenotype correlation · Heterogeneous disease · Homozygosity mapping · Mutation · Population · Retinitis pigmentosa

19.1 Introduction

Inherited retinal diseases (IRDs) are heterogeneous groups of disorders causing visual loss mainly due to degeneration of photoreceptors. IRDs include rod-dominated diseases such as retinitis pigmentosa (RP) in which rod photoreceptor loss precedes cone involvement, cone-dominated diseases such as cone-rod degeneration (CRD) in which cone photoreceptor loss precedes rod involvement, and regional degenerations such as Stargardt disease in which a specific retinal region is degenerated. The total number of genes responsible for these heterogeneous dis-

M. Hanany · D. Sharon (✉)
Department of Ophthalmology, Hadassah-Hebrew University Medical Center, Jerusalem, Israel
e-mail: dror.sharon1@mail.huji.ac.il

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eases is still unknown but is estimated to be over 300 (RETNET database at https://sph.uth.edu/retnet/). Heterogeneity is an important feature of IRDs at both the genetic and clinical aspects, and IRDs are considered the most heterogeneous group of inherited diseases in humans. Heterogeneity complicates genetic counseling and testing since the mode of inheritance and potential causative genes are difficult for interpretation in many cases. The genetic cause of various retinal phenotypes in the Israeli and Palestinian populations has been reported so far (Table 19.1 and Fig. 19.1a), with RP, Usher syndrome (USH), achromatopsia (ACHM), and Leber congenital amaurosis (LCA), being the most common phenotypes.

19.2 The Structure of the Israeli and Palestinian Populations

The Israeli and Palestinian populations have unique structures and include different ethnic groups including Jews of various origins, Arab Muslims, Bedouins, Arab Christians, and Druze. Consanguinity and intra-community marriages have been reported to be high in different ethnic groups for many generations, stemming from historic, ethnic, religious, and cultural causes that shaped the genetic landscape of the populations and contributed dramatically to the relatively high prevalence of autosomal recessive (AR) diseases, including ocular diseases, in these populations. [30, 31, 83]

19.3 Inheritance Patterns of IRDs

IRDs can be inherited in different patterns, depending mainly on the population history and structure. About 50% of families include a single affected individual, and therefore it is not possible to determine the inheritance pattern prior to gene identification, although most cases are likely to be AR. In the USA and Europe, the most common inheritance patterns in families with at least two affected individuals are AR (50–60%), autosomal dominant (AD, 30–40%), and X-linked (XL, 5–15%) [84]. Due to the relatively high rates of consanguinity and intra-community marriages in the Israeli and Palestinian populations, the AR inheritance pattern is much more common reaching 80% in the vicinity of Jerusalem and even over 90% in the Arab-Muslim population [31]. A summary of all reported inheritance patterns is depicted in Fig. 19.1b clearly emphasizing the high prevalence of the AR inheritance pattern (92%).

19.4 Prevalence of IRDs

The prevalence of IRDs in the Israeli and Palestinian populations has been published for nonsyndromic RP [31] and achromatopsia [30] and is available for congenital stationary night blindness (CSNB- Sharon, unpublished data). In all cases, disease prevalence in the Israeli and Palestinian populations was found to be higher than reported in other populations that are known to have lower rates of consanguineous marriages. The prevalence of nonsyndromic RP in the vicinity of Jerusalem was found to be 2.5–3 times higher compared to the European and American populations. Similarly, the prevalence of achromatopsia among Arab Muslims who reside in the vicinity of Jerusalem was found to be about 1:5000 individuals, which is much higher than the prevalence (1:30,000–1:50,000 individuals) reported in various populations. Only one population, the Pingelap Island population, was reported to have a higher achromatopsia prevalence of 4–10% [85] due to a single founder mutation in the *CNGB3* gene. The interesting story of this population and the effect of the disease on their daily life are well described in *The Island of the Colorblind* book by Oliver Sacks.

19.5 Genetic Analyses of IRDs

Various methods have been used along the last 30 years to aid in the identification of the genetic cause of IRDs, including the traditional posi-

Table 19.1 A list of IRD genes reported to cause retinal diseases in the Israeli and Palestinian populations

Gene name	Inheritance pattern	Disease	Number of families	Relevant publication	Novel identified genes		Genetic analysis method
					Novel as IRD gene	Novel as nonsyndromic IRD gene	
ABCA4	AR	STGD	1	[1]			Linkage and HM
			6	[2]			Gene screening
ACO2	AR	Infantile cerebellar-retinal degeneration	2	[3]	+		HM and WES
ADAM9	AR	CRD	2	[4]	+		HM
		RP	2	[5]			WES
AIPL1	AR	LCA	5	[6]			Gene screening
			1	[5]			WES
ALMS1	AR	CRD	1	[7]			WES
ARL2BP	AR	RP	1	[8]	+		HM and WES
ARL6	AR	BBS	1	[9]	+		Positional-candidate approach
ARMC9	AR	JS	1	[10]	+		MIPs and NGS
BBS1	AR	RP	1	[5]			WES
BBS2	AR	RP	4	[11]		+	WES
BBS4	AR	BBS	1	[12]			Gene screening
BBS7	AR	BBS	1	[12]			Gene screening
BEST1	AR	Best	1	[13]			Gene screening
CDHR1	AR	RD	1	[14]			WES
			1	[15]			WES
			1	[5]			WES
		RP	1	[5]			WES
		CRD	1	[16]			HM
CDH3	AR	HJMD	4	[17]	+		HM
			1	[18]			Gene screening
			5	[19]			Gene screening
			1	[20]			Gene screening
			2	[21]			Gene screening
			1	[22]			Gene screening
		EEM	1	[23]			Gene screening
		RP	1	[5]			WES

(continued)

Table 19.1 (continued)

Gene name	Inheritance pattern	Disease	Number of families	Relevant publication	Novel identified genes		Genetic analysis method
					Novel as IRD gene	Novel as nonsyndromic IRD gene	
CEP78	AR	CRD + SNHL	5	[24]	+		HM and WES
CEP250	AR	USH	1	[25]	+		HM and WES
CEP290	AR	LCA	1	[26]			HM
CERKL	AR	RD	7	[27]			HM
CHM	XL	CHM	1	[28]			WES
CNGA1	AR	RP	2	[5]			WES
CNGA3	AR	ACHM	10	[29]			Gene screening
			41	[30]			Gene screening
CNGB1	AR	RP	1	[5]			WES
			1	[31]			Gene screening
CNGB3	AR	ACHM	8	[30]			Gene screening
CNNM4	AR	Jalili syndrome	2	[32]	+		Positional-candidate approach
			1	[5]			WES
CRB1	AR	LCA	1	[33]			HM
			8	[34]			Gene screening and HM
		Early RP	1	[35]			Linkage analysis
			5	[34]			Gene screening and HM
			1	[31]			Gene screening
		RP	5	[34]			Gene screening and HM
CYP4V2	AR	RP	1	[5]			WES
C2ORF71	AR	RP	1	[36]	+		HM
			1	[25]			WES
			1	[5]			WES
C8ORF37	AR	RP	2	[37]	+		HM and NGS
			1	[15]			WES
DHDDS	AR	RP	9	[38]	+		HM
			7	[31]			Founder screening

EYS	AR	RP	10	[39]			HM
			1	[26]			HM
			3	[31]			Founder screening
			1	[28]			WES
FAM161A	AR	RP	20	[40]	+		HM
			1	[41]			HM
			1	[5]			WES
			3	[31]			Founder screening
GUCY2D	AR	LCA	4	[6]			Gene screening
			1	[42]			HM
	AD	CRD	6	[15]			WES
HGSNAT	AR	RP	2	[43]		+	WES
IDH3A	AR	RP	1	[44]	+		WES
IMPG2	AR	RP	2	[45]	+		HM
KCNV2	AR	CDSRR	5	[46]			Gene screening
LCA5	AR	LCA	1	[26]			HM
MAK	AR	RP	1	[47]	+		HM and WES
			1	[5]			WES
			2	[31]			Founder screening
MFRP	AR	RP	1	[26]			HM
MTTP	AR	Abetalipoproteinemia	6	[48]			Gene screening
MYO7A	AR	USH1	15	[49]			Gene screening
			2	[50]			HM
			1	[51]			HM and WES
			2	[28]			WES
NPHP4	AR	SLS	1	[28]			WES
NRL	AR	RP	1	[5]			WES
		ESCS	2	[52]			Gene screening
NR2E3	AR	ESCS/GFS/RP	10	[53]			Gene screening
			2	[26]			HM
			1	[5]			WES
PAX6	AD	Aniridia	1	[54]			Gene screening
			1	[55]			Gene screening
PCDH15	AR	USH1	8	[56]			Linkage
			2	[57]			Gene screening

(continued)

Table 19.1 (continued)

Gene name	Inheritance pattern	Disease	Number of families	Relevant publication	Novel identified genes		Genetic analysis method
					Novel as IRD gene	Novel as nonsyndromic IRD gene	
PDE6A	AR	RP	1	[5]			WES
PDE6B	AR	RP	1	[51]			HM and WES
PDE6G	AR	Early RP	1	[58]	+		HM
PEX6	AR	Zellweger syndrome	1	[59]			Gene screening
		Heimler syndrome	1	[60]			Gene screening
PLA2G5	AR	Late-onset RP	1	[5]			WES
PRCD	AR	RP	9	[61]			HM
PROM1	AR	CRD	1	[62]			HM
			1	[26]			HM
			1	[63]			HM
PRPF3	AD	RP	1	[5]			WES
RAB28	AR	CRD	1	[64]	+		HM and WES
RDH5	AR	FAP	14	[65]			Gene screening
			1	[5]			WES
RDH12	AR	Early RP	1	[35]			HM
			7	[26]			HM
			4	[5]			WES
			1	[31]			Founder screening
RHO	AD	RP	9	[66]			Gene screening
OPN1LW/ OPN1SW	XL	BCM	6	[67]			Gene screening
RPE65	AR	LCA	10	[6]			Gene screening
			1	[5]			WES
		RP	2	[5]			Gene screening
RPGR	XL	RP	1	[68]			Gene screening
			6	[31]			Gene screening
			2	[28]			WES
RP1	AR	RP	1	[5]			WES
RP2	XL	RP	1	[31]			Gene screening
SCAPER	AR	RP	1	[69]	+		WES
SLC38A8	AR	Foveal hypoplasia	3	[70]			HM and WES

SPATA7	AR	LCA	1	[5]			WES
		Early RP	1	[71]			HM
TRIM32	AR	BBS	1	[72]	+		Positional-candidate approach
TSPAN12	AR	FEVR	1	[5]			WES
			1	[73]			WES
TULP1	AR	LCA	1	[74]			Linkage
			2	[26]			HM
			1	[5]			WES
USH1C	AR	RP	9	[75]		+	WES
USH2A	AR	USH2	3	[76]			Gene screening
			2	[77]			Gene screening
			9	[78]			Gene screening
			1	[79]			Gene screening
		USH2, RP	1	[77]			Gene screening
		RP	2	[5]			WES
			1	[31]			Gene screening
USH3A	AR	USH3	5	[80]			Linkage and gene screening
			11	[81]			Gene screening
			3	[82]			Gene screening
USH1G	AR	USH1	1	[50]			WES

ACHM achromatopsia, *BBS* Bardet-Biedl syndrome, *BCM* blue cone monochromatism, *CDSRR* cone dystrophy with supernormal rod response, *CHM* choroideremia, *CRD* cone-rod degeneration, *EEM* ectodermal dysplasia, ectrodactyly, and macular dystrophy, *ESCS* enhanced S-cone syndrome, *FAP* fundus albipunctata, *FEVR* familial exudative vitreo-retinal disease, *GFS* Goldmann-Favre syndrome, *HJMD* congenital hypotrichosis associated with juvenile macular dystrophy, *HM* homozygosity mapping, *JS* Joubert syndrome, *LCA* Leber congenital amaurosis, *MIPs* molecular inversion probes, *RD* retinal degeneration, *RP* retinitis pigmentosa, *SLS* Senior-Loken syndrome, *SNHL* sensorineural hearing loss, *STGD* Stargardt disease, *USH* Usher syndrome, *WES* whole exome sequencing

tional cloning approach that is based on genetic linkage, the gene candidate approach, homozygosity mapping, and whole exome sequencing (WES). The approach to be used is determined by multiple factors and mainly the population structure, clinical phenotype, and inheritance pattern. In many Asian populations, consanguinity rates are relatively high, and therefore the homozygosity mapping approach was a major tool for gene identification. In this method, the genome of a patient is analyzed by studying the genotype of a large number of polymorphisms allowing one to determine which genomic regions are at a homozygous state. Such regions are highly likely to contain the causative mutation not only in consanguineous families but also in families of intra-community marriages. Disease-causing mutations have been reported thus far in 74 genes in the Israeli and Palestinian populations (Table 19.1), with *CNGA3* and *FAM161A* being the causative genes in the largest number of reported families (Fig. 19.1c). Most of the studies have been reported since 2007 (Fig. 19.2- red graph). Homozygosity mapping was indeed a valuable tool for gene identification (Table 19.1) and was used as the major mapping technique or in conjunction with other methods such as WES, to more accurately locate the causative gene.

19.6 Identification of Novel IRD Genes

Due to the large number of genes in which mutations can cause IRDs, each studied population shows a different proportion of contributing genes. Therefore, newly studied populations have a high probability to aid in the identification of novel disease-causing genes.

Studying Israeli and Palestinian IRD families resulted so far in the identification of 23 novel genes: 20 that were not previously associated with a retinal disease and 3 that were initially associated with syndromic IRD and later were found to cause a nonsyndromic IRD phenotype, usually in a genotype-phenotype correlation (Table 19.1). The vast majority of novel genes have been reported during the last 7 years (Fig. 19.2- green bars), mainly using next-generation sequencing techniques, such as WES. Although most of the novel genes were identified by analyzing a small number of Israeli and Palestinian families, some were found to be major IRD genes in these populations, including *CDH3* (16 families), *FAM161A* (25 families), and *DHDDS* (16 families), and all three have been identified using the homozygosity mapping approach. *FAM161A*, for example, has been identified by performing homozygosity mapping on a large number of consanguineous families with RP leading to the identification of a large homozygous region on chromosome 2 [40], overlapping with a previously reported RP locus (RP28). Screening all genes within the interval led to the identification of three null mutations in *FAM161A*, which was not considered as a good candidate prior to mutation screening. Similarly, *DHDDS* has been identified by homozygosity mapping in small families of Ashkenazi Jewish origin and gene prioritization [38].

The identification of *PDE6G* as a novel cause of early-onset RP [58] is unique and tangled with the history of the Arab-Muslim population in North Israel, who live in small towns, which were originally settled by a small number of related individuals. Therefore, most of the genetic diseases frequent among Israeli Arabs are due to founder effects. Using genome-wide homozygosity mapping, Dvir and colleagues [58] identified a homozygous 4.7-Mb region on chromosome 17q25.3 in patients who reside in the same town and suffer from early-onset RP. The authors then applied the candidate approach and identified an excellent candidate gene within the linked region: *PDE6G* which encodes for the inhibitory γ subunit of rod photoreceptor cyclic GMP phosphodiesterase. A homozygous canonical splice-site mutation was then identified using Sanger sequencing of all coding exons. Interestingly, although the mutation was missing in 256 Arab-Muslim controls, a relatively high percentage of controls who reside in the same village (7 out of 84, carrier frequency of 8.3%) were found to be heterozygous for this mutation.

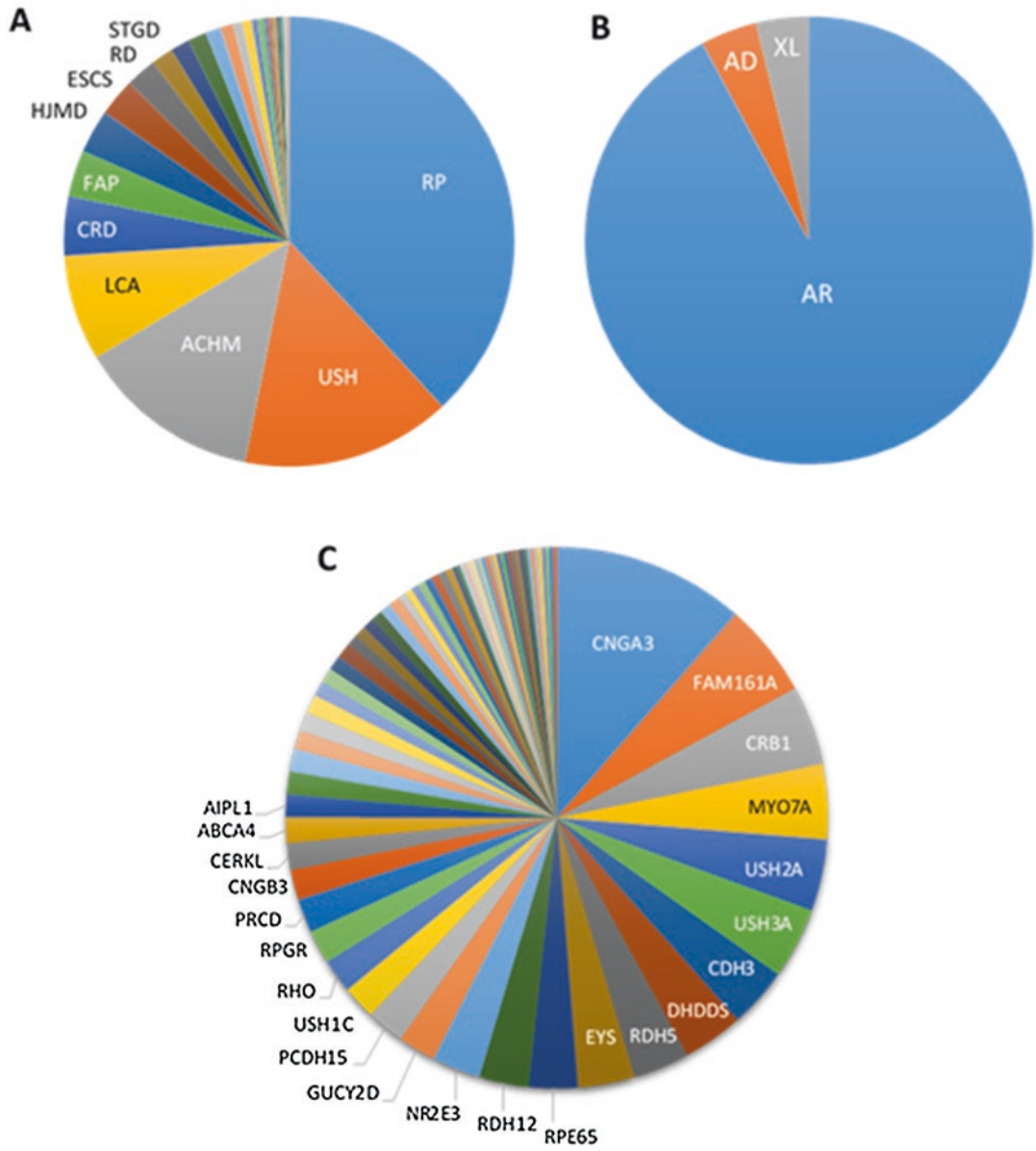

Fig. 19.1 Pie chart analyses of IRD characteristics in the Israeli and Palestinian populations. (**a**) Different IRD phenotypes. (**b**) IRD Inheritance patterns. (**c**) IRD genes in which mutations were reported in the Israeli and Palestinian populations. (See also Table 19.1)

19.7 Founder Mutations and Their Importance

Founder mutations are pathogenic variants observed in a group of individuals that is or was geographically or culturally isolated, in which at least one of the ancestors carried them. The relatively frequent existence of an AR disease in an isolated population usually suggests a founder effect. The Arab-Muslim and Jewish populations in Israel and the Palestinian territories are speculated, mainly by historical records, to originate from a single founder population, with an estimated divergence time of a few thousand years.

Table 19.2 IRD-causing founder mutations in the Israeli and Palestinian populations

Gene	Mutation name	Number of families	Subpopulation	References
CNGA3	p.Val529Met	9	AMJ	[29, 30]
		4	OJ	[29]
	p.Ile314del	12	AMJ	[30]
DHDDS	p.Lys42Glu	16	ASH	[31, 38]
EYS	p.Thr135Leufs*26	13	NAJ	[26, 31]
FAM161A	p.Arg523*	5	Mainly NAJ	[40]
	p.Thr452Serfs*3	19		[5, 31, 40]
MYO7A	p.Ala826Thr and IVS4+2T>G	7	NAJ	[49]
PCDH15	p.Arg245*	10	ASH	[56, 57]
PRCD	p.Arg22*	9	AMO	[61]
RPE65	IVS2-2A > T	10	NAJ	[6]
USH1C	p.Gly407Glufs*58	9	YJ	[75]
USH3A	p.Asn48Lys	18	ASH	[80–82]

AMJ Arab Muslims (vicinity of Jerusalem), *AMO* Arab Muslims (other areas), *ASH* Ashkenazi Jews, *NAJ* North African Jews, *OJ* Oriental Jews, *YJ* Yemenite Jews

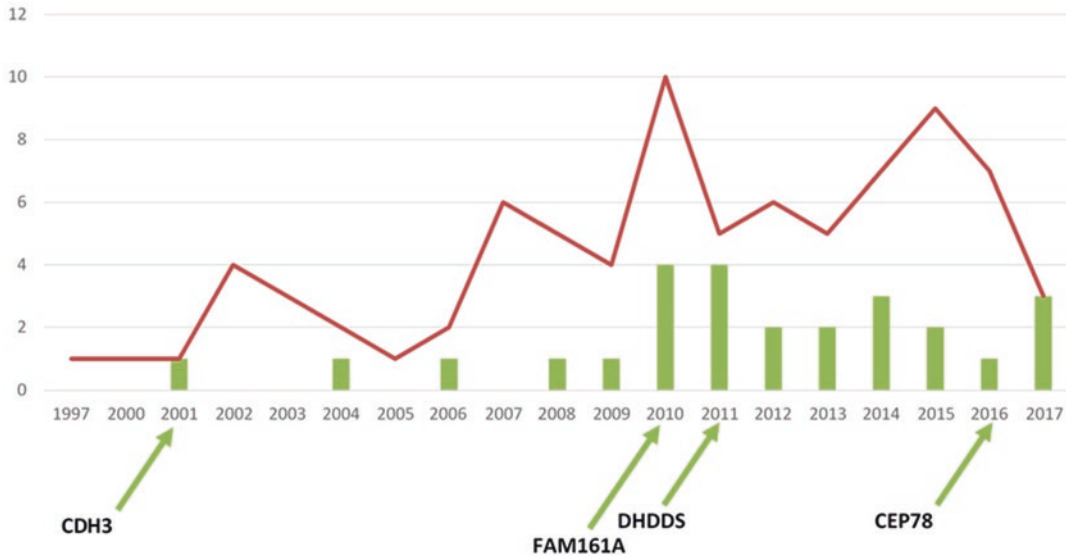

Fig. 19.2 IRD-related publications and novel genes reported by analyzing Israeli and Palestinian families. The graph shows the number of publications (red line) and novel genes (green bars) reported between the years 1997 and 2017. The most common novel genes identified by studying the Israeli and Palestinian populations are marked by arrows

Founder mutations identified in the Israeli Jewish population, and mainly in Ashkenazi Jews, were also reported to be prevalent among non-Israeli Jews residing in North America [38, 86]. At least 40 Mendelian disorders have been described in various Jewish subpopulations [87]; for some disorders, each of these subpopulations has its own unique set of founder mutations. The Ashkenazi Jewish population segregates a variety of genetic conditions caused by prevalent founder mutations, including Tay-Sachs disease, cystic fibrosis, and Usher syndrome [87]. Founder mutations in the Israeli population are usually restricted to a specific ethnic group, e.g., the

USH3A, p.Asn48Lys mutation in Ashkenazi Jews [81], or the *CERKL*, c.238+1G>A mutation among patients of Jewish Yemenite origin [27]. On the other hand, some mutations have been identified in multiple Jewish ethnic groups, including two founder mutations in *FAM161A* that were identified in Jews from three different origins (Ashkenazi Jews, Oriental Jews, and North African Jews) [40]. These mutations were not reported so far in other populations.

Interestingly, a few disease-causing mutations are shared among the Arab-Muslim and the Jewish populations, most of which are pan-ethnic and appear in almost every studied population (e.g., the p.Gly1961Glu variant in *ABCA4*). However, one *CNGA3* mutation tells a highly interesting story [29]. The c.1585G>A (p.Val529Met) mutation in this gene has been reported in Arab Muslims from the vicinity of Jerusalem, Oriental Jews, as well as a few European families. Haplotype analysis demonstrated that the European alleles have at least two different origins that are clearly different from the one that is shared by the Arab Muslims and Oriental Jewish patients who carried this allele. This shared haplotype was relatively large, spanning 21.5 cM (~11 Mbp) with an estimated age of about 5000 years, indicating a common origin of these two ethnic groups.

A classical founder mutation in the Arab-Muslim population is the one reported in the *PRCD* gene [61]. Prior to this study, only a single patient (residing in Bangladesh) has been reported to suffer from RP due to a *PRCD* mutation. By analyzing a large set of patients (18 individuals who belong to nine families) residing in the same Arab-Muslim village, the authors identified a homozygous nonsense mutation in *PRCD* with a high carrier frequency (10%) in normal controls who reside in the same village. These results therefore provide a strong and important confirmation for the role of *PRCD* in the etiology of RP in humans.

The identification and characterization of such common mutations in a specific ethnic population allows sensitive and specific use of genetic testing for carrier screening, genetic counseling, and diagnostic purposes.

19.8 Genotype-Phenotype Correlations

Different mutations within the same gene usually result in the same phenotype. Out of the 74 genes listed in Table 19.1, mutations in 63 of the genes (85%) have been described to cause a single phenotype in the studied populations. On the other hand, a few unique cases show variable phenotypes that can be mutation-dependent.

An example of a clear genotype-phenotype correlation has been reported in the *USH1C* gene [75]. Dozens of different mutations in *USH1C* are known to cause Usher syndrome type 1 (congenital sensorineural deafness, vestibular dysfunction, and RP). However, using a combined approach of homozygosity mapping and WES, a homozygous frameshift mutation has been identified in 16 patients of a Yemenite Jewish origin with RP and either normal hearing or late-onset (after the age of 40 years) mild-to-severe hearing loss. What is special about this particular mutation that does not result in severe early-onset deafness? Similar to other genes in the human genome, *USH1C* can produce at least four different proteins through the alternative splicing mechanism. The identified mutation (c.1220del; p.Gly407Glufs*58) is located in an alternative exon (#15) that is mainly expressed in the retina, and therefore loss of this isoform leads mainly to retinal degeneration.

Mutations in some genes, however, can lead to variable phenotypes, with no clear genotype-phenotype correlation. The best example is probably the c.238+1G>A (IVS1+1G>A) mutation in the *CERKL* gene [27]. This founder mutation is relatively common among patients of Yemenite Jewish origin with a carrier frequency of 4.4%. Interestingly, among the 24 patients who shared the same *CERKL* genotype (homozygous for c.238+1G>A), some were diagnosed with RP, while others were diagnosed with cone-rod degeneration (CRD). Electroretinography (ERG) analysis of the patients did not help in clarifying the clinical dilemma, as often a similar degree of rod and cone dysfunction was present rather than preferential involvement of one photoreceptor type or the other [27]. In addition, marked variability

in ERG responses has been noted among patients, similar to the variability reported among patients who carry mutations in the same gene. There is currently no clear explanation for such variability, but environmental factors as well as modifier genes might contribute to this phenomenon.

19.9 Therapeutic Modalities in IRDs

There is currently no cure for the vast majority of IRD types; however recent parallel advances in various fields bring new hope for curing or at least delaying the degeneration process in the near future. One of these modalities is gene augmentation therapy in which an adeno-associated virus (AAV) containing a normal copy of the gene (in which mutations are the cause of disease) is injected into the subretinal space. Three Israeli patients who are homozygous for a founder *RPE65* splicing mutation underwent this treatment [6], leading to an increase in vision in the treated area as early as 15 days after the intervention. An interesting story in this regard is the *CNGA3* gene. While in Europe and in the USA, mutations in *CNGB3* are the major cause of achromatopsia, in the Israeli and Palestinian populations, two major founder mutations in *CNGA3* make this gene the major cause of the disease (84% of solved achromatopsia families) [30]. A careful clinical assessment of *CNGA3* patients showed that in over 50% of patients, rods are also involved and that under dark- and light-adapted conditions, patients use rod-mediated pathways. This study determined the efficacy outcome measures for gene augmentation therapy of *CNGA3* that will include chromatic light-adapted psychophysics, with attention to the photoreceptor basis of the response, and quantitation of photoaversion. In parallel to this study, a homozygous *CNGA3* mutation was also found to cause achromatopsia in Israeli sheep flock that was successfully used for gene augmentation therapy [88].

Supplementary of vitamin A and its derivatives have been studied for many years as potential treatments for slowing down the process of photoreceptor degeneration [89]. Aiming to study the effect of 9-cis β-carotene on progression of retinal degeneration, 29 patients with RP of unknown etiology were treated with 9-cis β-carotene in a randomized crossover trial [90]. Although no improvement was evident in visual acuity, treated patients had better retinal function based on ERG testing.

As new treatment modalities are constantly being developed in Israeli research laboratories, including stem-cell therapy, retinal implants, nanotechnology, and artificial vision, this field is likely to progress rapidly during the coming 5–10 years.

19.10 Future Perspective

Identification of the vast majority or even all genes that cause IRDs in the Israeli and Palestinian populations is a matter of time. As DNA analysis dramatically improves and international collaborations become the prominent way of collaboration, the route for identifying all genes and mutations is relatively short. It is likely to assume that all major causative genes have been identified in populations that were studied during the last 10 years and the remaining genes are likely to affect a relatively small number of families worldwide, making international collaborations across countries and continents crucial to prove the pathogenicity of mutations identified in novel genes. Consortia like the European Retinal Disease Consortium (ERDC, http://www.erdc.info) and the Asian Eye Genetics Consortium (AEGC, http://www.asianeyegenetics.org) are likely to enhance this process dramatically. This progress will lead to more focused efforts in developing therapeutic modalities for IRDs, including gene- and even mutation- specific therapies (such as gene augmentation therapy and translational read-through therapy) as well as general therapeutic modalities, such as stem-cell therapy. It is clear that not a single therapy will cure or improve vision in all patients, but different modalities will be needed to be tailored to different stages of diseases and different etiologies.

As the carrier frequency for IRD mutations is predicted to be relatively high (1 out of 5–6 individuals), new offsprings will be born with genetic mutations that will cause disease, unless a treatment is applied. Although it is difficult to predict the time needed to develop therapy for most IRD types, both the large number of different treatments that are being developed and the large number of teams involved in this important effort are likely to yield results within a decade or two. Additionally, with proper genetic counseling for families with IRD-causing mutations, it is likely that the prevalence of IRDs will decline within 1–2 generations.

Compliance with Ethical Requirements Mor Hanany and Dror Sharon declare that they have no conflict of interest.

No human or animal studies were performed by the authors for this article.

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Occult Macular Dystrophy (Miyake's Disease)

20

Kazushige Tsunoda

Abstract

Occult macular dystrophy (OMD; OMIM 613587), first described by Miyake et al. in 1989, is an inherited macular dystrophy characterized by a progressive decrease in the visual acuity in eyes with a normal appearing fundus and normal fluorescein angiograms (Miyake Y, Ichikawa K, Shiose Y, Kawase Y, Am J Ophthalmol,108:292–9, 1989). The full-field electroretinograms (ERGs) are usually normal; however the focal macular ERGs, multifocal ERGs, and pattern ERGs are abnormal. Heterozygous mutations in the *retinitis pigmentosa 1-like 1 (RP1L1)* gene (OMIM 608581) cause this ocular condition, (Akahori M, Tsunoda K, Miyake Y, et al, Am J Hum Genet, 87:424–9, 2010, Tsunoda K, Usui T, Hatase T, et al, Retina J Retinal Vitreous Dis 32:1135–47, 2012, Miyake Y, Tsunoda K, Jpn J Ophthalmol, 59:71–80, 2015) and OMD with the *RP1L1* mutations has been specifically designated as Miyake's disease (Fujinami K, Kameya S, Kikuchi S, et al, Invest Ophthalmol Vis Sci, 57:4837–46, 2016). Characteristic changes in the microstructure of the photoreceptors have been detected by spectral-domain optical coherence tomography (SD-OCT) in eyes with Miyake's disease (Tsunoda K, Usui T, Hatase T, et al, Retina J Retinal Vitreous Dis 32:1135–47, 2012, Fujinami K, Kameya S, Kikuchi S, et al, Invest Ophthalmol Vis Sci, 57:4837–46, 2016). The most common mutation is the c.133C > T, p.Arg45Trp mutation in exon 2, and there is another hot spot between amino acid numbers 1194 and 1201 in exon 4 which is downstream of the doublecortin domain (Fujinami K, Kameya S, Kikuchi S, et al, Invest Ophthalmol Vis Sci, 57:4837–46, 2016, Kabuto T, Takahashi H, Goto-Fukuura Y, et al, Mol Vis, 18:1031–9, 2012, Davidson AE, Sergouniotis PI, Mackay DS, et al, Hum Mutat, 34:506–14, 2013). In addition to the typical phenotype of OMD, extensive retinal dysfunction such as generalized cone dysfunction and generalized rod-cone dysfunction has been documented in patients with biallelic mutations in the *RP1L1* gene (Davidson AE, Sergouniotis PI, Mackay DS, et al, Hum Mutat, 34:506–14, 2013, Kikuchi S, Kameya S, Gocho K, et al, Biomed Res Int, 2015:545243, 2015).

Keywords

Occult macular dystrophy · Miyake's disease · *RP1L1* gene · Progressive occult maculopathy

K. Tsunoda (✉)
Division of Vision Research, National Institute of Sensory Organs, Tokyo, Japan
e-mail: tsunodakazushige@kankakuki.go.jp

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20.1 Introduction

Occult macular dystrophy (OMD; OMIM 613587) is an inherited macular dystrophy characterized by a progressive decrease in the visual acuity in eyes with an essentially normal appearing fundus and normal fluorescein angiograms [1]. The important signs of OMD are normal full-field electroretinograms (ERGs) but abnormal focal macular ERGs (FMERGs), multifocal ERGs (mfERGs), and pattern ERGs. These findings indicate that the retinal dysfunction was confined to the macula. Since the initial report by Miyake et al. in 1989, there have been a number of reports describing the phenotype of this disorder, but the clinical characteristics varied in different reports [9–12]. In 2010, our laboratory found that dominant mutations in the *RP1L1* gene were responsible for OMD, [2–4] and a number of mutations in the *RP1L1* gene have been recently reported in patients of various ethnicities [5–7]. The genotypic and phenotypic investigations have confirmed that patients with OMD harboring *RP1L1* mutations, Miyake's disease, have homogeneous clinical characteristics and should be strictly segregated from macular dysfunction without genetic cause [3–5]. In this review, the clinical and genetic characteristics of Miyake's disease will be presented.

20.2 Clinical Features

20.3 Clinical Course of OMD Caused by *RP1L1* Gene Mutation

Patients with OMD initially complain of blurred vision, color vision abnormalities, and photophobia. Most of the patients with *RP1L1* mutations have a family history of dominant inheritance. The age of onset varies from 6 to 60 years, and the onset of the visual disorder may be different in the two eyes in some cases. Patients are generally affected in both eyes, and the visual acuity gradually decreases for a long duration which can range from 10 to 30 years [3]. In particular cases with central foveal sparing, patients remain asymptomatic although they have dysfunction of photoreceptors at the parafoveal regions [13]. There are only a few cases where the decimal visual acuity decreases to less than 0.1 even in senile patients with a long duration of OMD. The localized macular dysfunction can be confirmed either by Goldmann perimetry or automated static perimetry as a relative central scotoma. In patients examined shortly after the onset, a relative central scotoma is not detected by Goldmann perimetry but can be detected by static perimetry. Due to the normal fundus appearances and normal full-field ERGs, OMD is often misdiagnosed as optic neuropathy of unknown origin, amblyopia, or nonorganic visual loss. There are also cases with senile cataract, which can be later diagnosed as OMD due to unexpectedly low visual acuity following cataract surgery.

20.4 Retinal Appearances

The ophthalmoscopic appearances, fluorescein angiography (FA), indocyanine green angiography, and fundus autofluorescence (AF) are normal in OMD [3, 4] (Fig. 20.1). In some cases with long duration, round-shaped very weak staining at the fovea may be present in FA.

The AF images are generally normal in the entire posterior pole region. However, some cases (~50%) may demonstrate a round-shaped areas of increased AF signals at the fovea [14]. The round-shaped areas of increased AF are very faint in some cases and more apparent in others. The relationship between duration of the disease and intensity of the increased AF signal has not been determined.

20.5 Optical Coherence Tomography (OCT)

Spectral-domain OCT is a very important method in the diagnosis of OMD [3, 13, 14]. In patients with the *RP1L1* mutation, the most prominent features on OCT are the abnormalities of the two

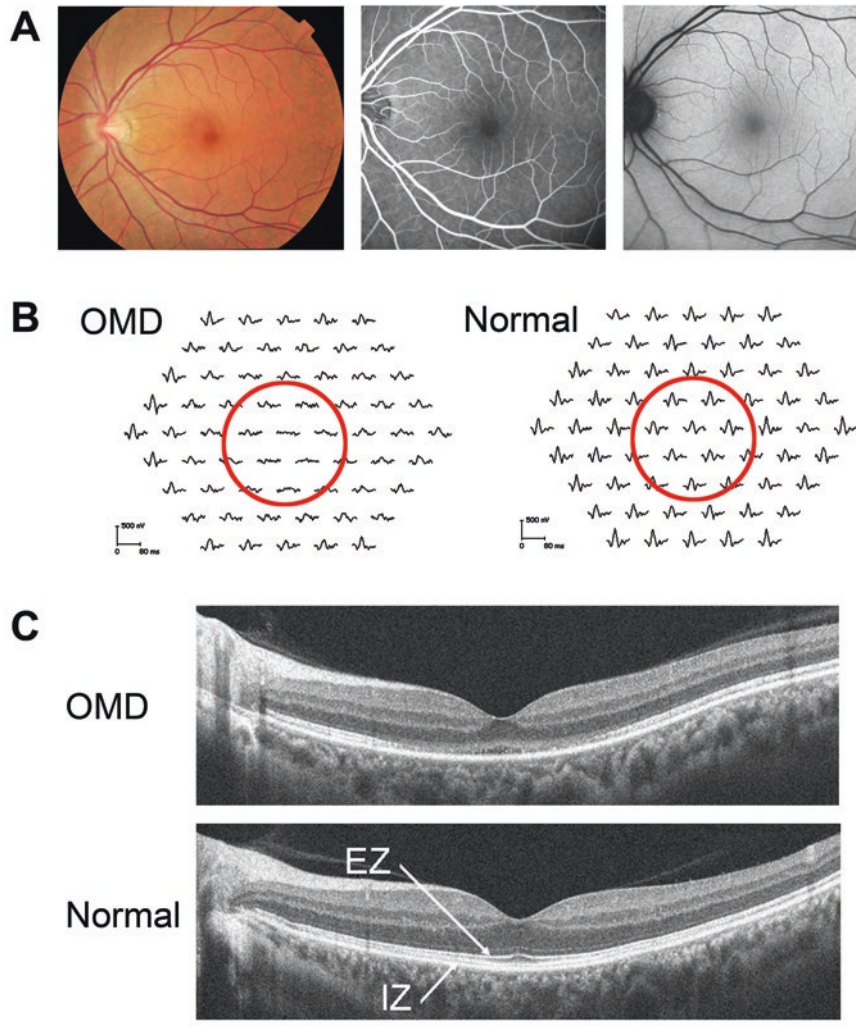

Fig. 20.1 Fundus photographs, multifocal electroretinograms (mfERGs), and optical coherence tomographic (OCT) images of a patient with a *RP1L1* mutation (p. Arg45Trp, heterozygous). (**a**); Fundus photographs (left), fluorescein angiograms (center), and fundus autofluorescence (right), showing no abnormal findings. (**b**); Trace arrays of mfERGs tested with 61 hexagonal stimuli in an eye with OMD (left) and normal eye (right). The responses in the central loci are extinguished in the eye with OMD. (**c**); OCT images horizontally profiled along the foveola in OMD (top) and normal eye (bottom). The ellipsoid zone (EZ) appears blurred, and the interdigitation zone (IZ) is absent in the macular region in the eye with OMD

highly reflective lines in the OCT images at the macular region. These lines correspond to the ellipsoid zone (EZ) and interdigitation zone (IZ) in the photoreceptor layer (Fig. 20.1). The EZ at the fovea appears thickened and blurred in the early to middle stages of OMD and disrupted or absent in the later stages. The IZ cannot be clearly observed in the macular area even at the early stage of OMD. In the peri-macular regions which have normal visual function, these outer retinal microstructures appear normal. In longer duration cases, e.g., >30 years, both the photoreceptors and outer nuclear layers are thinnest at the fovea; however, the retinal pigment epithelium remains unchanged.

It is notable that there are asymptomatic family members who have *RP1L1* mutations in some families with dominant OMD [13]. The OCT

images of these asymptomatic cases demonstrated photoreceptor abnormalities only in the parafoveal regions, viz., absence of the IZ and blurring of the EZ. However, the microstructures of the photoreceptors are preserved at the foveal center. The sparing of the photoreceptor layer at the central foveal accounts for the well-preserved visual acuity in the asymptomatic patients. It has not been determined whether the sparing represents an initial phase of typical OMD or a subtype of macular lesion associated with OMD.

20.6 Electrophysiology

Electrophysiological tests are the key for the diagnosis of OMD. Both the scotopic (rod) and photopic (cone) responses of the full-field ERGs are normal; however the focal macular ERGs, multifocal ERGs, and pattern ERGs are abnormal even at the very early stage of the macular dysfunction in OMD patients[1, 4] (Fig. 20.1). The amplitudes of the cone-induced responses of the full-field ERGs may be borderline or slightly reduced in some cases with the *RP1L1* mutation, [15] where the region of dysfunctional retina expands over the macula toward the periphery. These cases may be better referred to as central cone dystrophy rather than macular dystrophy from the viewpoint of electrophysiology.

20.7 Genetics

Heterozygous mutations in the *retinitis pigmentosa 1-like 1 (RP1L1)* gene (OMIM 608581) cause this ocular condition, and OMD with the *RP1L1* mutations has been specifically designated as Miyake's disease [2, 3, 5]. The RP1L1 protein was suggested to be involved in the maintenance of the morphological and functional characteristics of the photoreceptors, [16, 17] and a number of mutations in the *RP1L1* gene have been reported [2, 3, 6, 7, 15, 18–21]. The most common mutation is the c.133C > T, p.Arg45Trp mutation in exon 2, [2, 3, 7, 15, 18, 20, 21] and there is another hot spot between amino acid numbers 1194 and 1201 in exon 4 which is downstream of the doublecortin domain [5–7]. The genetic background leading to the OMD may be a variant; however, other genetic causes contributing to this disease have not been determined. In addition to the typical phenotype of OMD, extensive retinal dysfunction such as generalized cone dysfunction and generalized rod-cone dysfunction has been documented in patients with biallelic mutations of the *RP1L1* gene [7, 8].

The *RP1L1* gene was originally cloned as a gene derived from common ancestors with the retinitis pigmentosa 1 (*RP1)* gene, which is responsible for 5–10% of all autosomal dominant retinitis pigmentosa (RP) worldwide. It is located on chromosome 8 [16, 22–25]. An immunohistochemical study on cynomolgus monkeys showed that *RP1L1* was expressed in rod and cone photoreceptors, and it is believed to play important roles in the morphogenesis of the photoreceptors [16, 17]. Heterozygous *RP1L1* knockout mice were reported to have normal retinal morphology, while homozygous knockout mice developed subtle retinal degeneration [17]. However, the RP1L1 protein has a very low degree of overall sequence identity (39%) between humans and mice compared to the average values of sequence similarities observed between human and mice proteins. The cellular mechanisms that explain why only the macular region is impaired in human OMD patients have not been determined.

20.8 Miyake's Disease and Occult Maculopathy

Patients with OMD having the *RP1L1* mutation have a similar clinical course and share the common OCT findings: blurred EZ and absence of IZ of the photoreceptors in the macular region. We categorized these patients as OMD with classical phenotype and those lacking at least one of these two features as nonclassical phenotype [5]. In a cohort throughout Japan, a significant association was found between the OCT phenotypes and molecular genotypes; OMDs with *RP1L1* mutations have classical phenotype, and those without *RP1L1* mutations have nonclassical phenotypes.

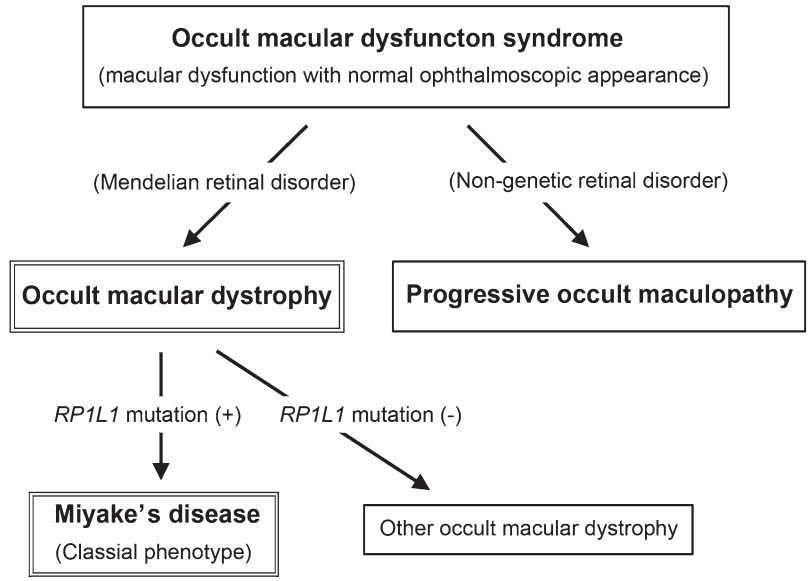

Fig. 20.2 Classification of occult macular dysfunction syndrome Occult macular dysfunction syndrome includes three subcategories: (**a**) *RP1L1*-associated occult macular dystrophy (Miyake's disease), (**b**) other hereditary occult macular dystrophy caused by other gene abnormalities, and (**c**) non-hereditary occult macular dystrophy-like syndrome (progressive occult maculopathy)

There are two types of pathophysiology associated with bilateral progressive central cone dysfunction with normal fundus [5] (Fig. 20.1). One is a Mendelian hereditary occult macular dystrophy caused by genetic abnormalities such as the *RP1L1* mutations (Miyake's disease) and possibly other unknown gene mutations, i.e., other occult macular dystrophies. The other type is a retinopathy with clinical signs of the occult macular dysfunction syndrome, i.e., clinical and ERG findings similar to hereditary occult macular dystrophy but not related to the Mendelian genetic abnormality. We refer to this non-Mendelian form as progressive occult maculopathy (Fig. 20.2).

Compliance with Ethical Requirements The procedures used adhered to the tenets of the Declaration of Helsinki, and approval to perform this study was obtained from the Review Board/Ethics Committee of the National Institute of Sensory Organs, National Hospital Organization, Tokyo Medical Center.

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Clinical Genetics of Vitelliform Macular Dystrophy: An Asian Perspective

21

Sung Wook Park, Chang Ki Yoon, Dae Joong Ma, Un Chul Park, and Hyeong Gon Yu

Abstract

Vitelliform macular dystrophy (VMD) is a group of macular dystrophy characterized by the subretinal accumulation of yellow yolk-like materials which predominantly affect the macula. Best vitelliform macular dystrophy is among the most common autosomal dominant (AD) retinal dystrophy, caused by mutations in the *BEST1* gene. Since first identification of *BEST1* gene in 1998, molecular biology and pathophysiology of *BEST1* gene and vitelliform macular dystrophy were studied. Recent advances in genetic analysis have described over 200 different human *BEST1* mutations to date, associated with a broad spectrum of ocular diseases, called bestrophinopathy. However, the genotype-phenotype correlation in VMD is largely unexplored. Genetic test is clinically important in the diagnosis of VMD because the clinical features of VMD are similar to those of exudative age-related macular degeneration (AMD), choroidal neovascularization (CNV), or central serous chorioretinopathy (CSC). Here, in addition to describing the clinical characteristics of VMD, this chapter focuses on the clinical genetics of *BEST1* gene in VMD.

Keywords

Vitelliform macular dystrophy · Bestrophin-1 · Best vitelliform macular dystrophy · Adult-onset vitelliform macular dystrophy · *BEST1* gene mutation · Genome editing

21.1 Introduction

Macular dystrophy is a group of heritable disorders that cause ophthalmoscopically visible macular abnormalities. Vitelliform macular dystrophy (VMD) is a group of macular dystrophy characterized by the subretinal accumulation of yellow yolk-like materials which predominantly affects the macula. Best vitelliform macular dystrophy (BVMD) is named after Friedrich Best who described a family with a history of early-onset macular degeneration in 1905 [1]. BVMD is among the most common autosomal dominant (AD) retinal dystrophy, caused by mutations in the *BEST1* gene. Since the first identification of

S. W. Park · D. J. Ma · U. C. Park · H. G. Yu (✉)
Department of Ophthalmology, Seoul National University College of Medicine, Seoul, Republic of Korea

Department of Ophthalmology, Seoul National University Hospital, Seoul, Republic of Korea

Retinal Degeneration Research Laboratory, Biomedical Research Institute, Seoul National University Hospital, Seoul, Republic of Korea
e-mail: hgonyu@snu.ac.kr

C. K. Yoon
Department of Ophthalmology, Inje University College of Medicine, Busan, Republic of Korea

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BEST1 gene in 1998 [2], molecular biology and pathophysiology of *BEST1* gene in VMD have been studied. Recent advances in genetic analysis have described over 200 different human *BEST1* mutations to date, associated with a broad spectrum of ocular diseases, called a bestrophinopathy [3, 4]. Bestrophinopathy includes five clinically distinct categories BVMD, adult-onset vitelliform macular dystrophy (AVMD), autosomal recessive bestrophinopathy (ARB), autosomal dominant vitreoretinochoroidopathy (ADVIRC), and retinitis pigmentosa. AVMD was first described by Gass in 1974 who initially termed it peculiar foveomacular dystrophy [5]. AVMD is one of the most common forms of macular dystrophy as well [6]. Many investigators suggested that AVMD is a mild form of BVMD within the same spectrum because the clinical features of AVMD were similar to those of early-stage BVMD and the age of onset were highly variable [7–9]. Clinically, BVMD is distinguished from AVMD by earlier age of onset, larger lesion size, and an abnormal electrooculogram (EOG). Clinical features of VMD are similar to those of exudative age-related macular degeneration (AMD), choroidal neovascularization (CNV), or central serous chorioretinopathy (CSC). Thus, genetic test is clinically important in the diagnosis of VMD. Here, in addition to describing the clinical characteristics of VMD, this chapter focuses on the clinical genetics of *BEST1* gene in VMD (BVMD and AVMD).

21.2 Epidemiology and Asian Perspective

VMD is an autosomal dominant macular dystrophy with an estimated prevalence of 1 in 10,000 in the USA [10], 2/10,000 in Sweden [11], 1.5/100,000 in Denmark [12], and 1 in 16,500 to 1 in 21,000 in Olmsted County, Minnesota, USA [13]. Males are more affected than females (3:1) [11, 12]. Despite the update of novel mutations of *BEST1* in Asian VMD patients, there was no report of the prevalence of VMD in Asian countries. Thus, a study of the prevalence of VMD with a genetic analysis in Asian countries is necessary.

21.3 Molecular Biology

The *BEST1* gene consists of 11 exons that encode the bestrophin-1 protein (585 amino acids). Bestrophin-1 is a retinal pigment epithelium (RPE) protein hypothesized to function as a Ca^{2+}-activated Cl^- channel (CaCC), or a regulator of ion transport [14]. Bestrophin-1 is predominantly expressed in the basolateral membrane of the RPE [15]. X-ray structure of chicken BEST1-Fab complexes indicates that Bestrophin-1 forms a homo-pentamer and functions as a CaCC [16]. Disease-causing mutations are prevalent within the gating apparatus. In addition, Bestrophin-1 functions as a regulator of intracellular calcium signaling and influences transepithelial electrical properties [17]. Recently, patient's stem cell-derived RPE is used for the function of bestrophin-1 and reveals that bestrophin-1 assembles into a key calcium-sensing chloride channel in human RPE [18]. Further study using RPE cells from patient-derived induced pluripotent stem cells (iPSc) harboring *BEST1* mutations is required to elucidate the exact functional role of bestrophin-1.

21.4 Clinical Features

21.4.1 BVMD

BVMD is an early-onset autosomal dominant disorder showing extremely variable penetrance and expressivity. The diagnosis of BVMD shows a bimodal age distribution; the first maximum peak was made during the childhood, but the second peak was made following puberty and extending into the sixth decade of life [19]. Before the era of genetic analysis, the diagnosis of BVMD was based on typical fundus findings, family history, and a decreased Arden ratio (light peak/dark trough) of EOG with a normal electroretinogram (ERG), which may contribute to variability of penetrance, expressivity, and onset age.

BVMD is caused by dysfunction of Bestrophin-1 protein, a CaCC protein located on the basolateral membrane of RPE, which causes abnormal fluid and ion exchange that decreases

pumping of fluid from the subretinal space, and results in swelling of RPE and subretinal lipofuscin accumulation [20]. Histopathologically, autofluorescent material was accumulated in the outer retina and the subretinal space in BVMD, which is considered as indigestible components of photoreceptor outer segments that accumulate due to the lack of direct apposition of the outer segments and the RPE [21]. Eventual phagocytosis of these older materials over time would load the RPE cells and may account for excessive accumulation of abnormal lipofuscin in RPE cells across the entire fundus [22]. These findings coincide with the decreased Arden ratio of EOG, less than 1.5, seen in BVMD, which suggest generalized dysfunction of the RPE. Even otherwise asymptomatic carriers of *BEST1* mutations will exhibit an altered EOG [23]. Full-field ERG is generally normal, but the multifocal ERG amplitudes of the central and pericentral responses were significantly reduced in the majority of patients [24]. However, the photoreceptor structure evaluated cellular imaging with adaptive optics scanning light ophthalmoscopy was retained within active BVMD lesions, even in apparently advanced disease [25, 26].

Five progressive stages can be defined based on fundus examination [20, 27]. However, these stages are not observed in all patients, nor do they occur consecutively. The first previtelliform stage is characterized by the absence of symptoms and subtle RPE changes such as RPE mottling and a small yellow spot. On optical coherence tomography (OCT), RPE and ellipsoid zone (EZ) disruption was detectable in a small fraction of eyes [28, 29]. A slight thickening of the interdigitation zone was also observed [30]. EOG is abnormal and fluorescein angiogram (FA) shows window defects. Visual acuity remains intact in most patients. The previtelliform lesions are characterized by absence or only slight autofluorescence on fundus autofluorescence (FAF) imaging.

The second vitelliform shows a well-circumscribed, circular, homogeneous, yellow-opaque, 0.5–3 disc diameter sized, yolk-like macular lesions. The remaining part of fundus usually has normal appearance, but multifocal lesions also can be seen. The accumulation of hyperreflective vitelliform material is clearly visible on OCT below the neurosensory retina, located between the EZ and the RPE. The disruption of outer retinal layers and neurosensory retinal detachment with subretinal fluid occur in many cases [28, 29]. The yellowish subretinal material is intensely hyperautofluorescent in FAF imaging. FA shows marked hypofluorescence in the zone covered by lesion by blockage of fluorescence. Metamorphopsia, blurred vision, and a decrease of central vision can occur.

In the third pseudohypopyon stage, the vitelliform material accumulates inferiorly and develops a fluid level. On OCT, the upper part of the lesion is observed as hyporeflective area located between RPE and EZ, with clumping of hyperreflective material on the posterior retinal surface. The lower part of the lesion, where the vitelliform material is still accumulated, shows a highly reflective area located in the subretinal space. FA shows hypofluorescence in the lower part resulted from the blockage by the vitelline material. The superior part shows hyperfluorescent due to transmission defects linked to RPE and chorioretinal atrophy in the early phase. FAF shows loss of autofluorescence, particularly in the upper part.

The fourth vitelliruptive stage is characterized by the partial reabsorption of the vitelliform material. This vitelliform material becomes less homogeneous to develop a "scrambled-egg" appearance. OCT shows an optically empty lesion between EZ and RPE, with clumping of hyperreflective material on the posterior retinal surface like the upper part of the pseudohypopyon. The areas of focal RPE hypertrophy can be observed as hyperreflective mottling on the RPE layer on some parts. FAF shows decreased of autofluorescence centrally but increased autofluorescence at the outer border of the lesion.

In the last atrophic/fibrotic stage, RPE atrophy and loss of central vision occur after rupture and reabsorption of the cystic lesion. FA shows hyperfluorescence without leakage. OCT reveals thinning of all the retinal layers and diffuse disappearance of outer retinal layers within the macular area, with highly hyperreflective thickening at the RPE level [29, 31]. Atrophic lesions are characterized by decreased autofluorescence on FAF.

Choroidal neovascularization (CNV) may develop and can lead to form a disciform scar. Patients usually underwent sudden visual disturbance with central scotoma and/or metamorphopsia, showing a macular hemorrhage on fundus examination. In that case, FA shows hyperfluorescence because of CNV and leakage. Intravitreal injection of anti-vascular endothelial growth factor (VEGF) agent was effective in treating CNV complicated with BVMD and safe even in children [32–34].

Patients with BVMD undergo a progressive decrease of vision over time. In a study that evaluated the course of visual decline of 53 patients in BVMD with *BEST1* mutation [35], the median age of onset of visual symptoms was 33 years. Twenty-five percent of patients retain visual acuity of 20/40 or better at the age of 66 years. Other study evaluated 47 patient with BVMD; 74% of patient older than 30 years had 20/100 or worse visual acuity at least one eye [36].

21.4.2 AVMD

Gass reported a three-generation family and six sporadic patients characterized by one-third disc diameter sized bilateral subfoveal vitelliform lesions with onset between the ages of 30 and 50 years accompanied by slowly progressive visual loss as "peculiar foveomacular dystrophy." They also showed occasional paracentral drusen, normal to slightly subnormal response on EOG but normal ERG and color vision [5]. AVMD shows a variable genetic inheritance, although most cases are sporadic [37]. Patients with AVMD may be asymptomatic but become symptomatic in the fourth or fifth decade of life with blurred vision, metamorphopsia, or scotoma and typically have slow progression of vision loss [38]. Patients with AVMD typically present a round, yellowish subretinal deposit in one-third to one disc diameter size within the macular area, similar fundus finding to the vitelliform stage of BVMD.

The initial yellow lesion may present in only one eye and appear as small yellow flecks in the paracentral area. EOG shows a normal or slightly reduced Arden ratio, which is obviously abnormal in BVMD. The macular lesion appears as hyperautofluorescent in FAF. The vitelliform deposit usually appears as initially hypofluorescent but gradually becomes hyperfluorescent on the edges by staining of the dye in FA [39] and hypofluorescent on indocyanine green angiography (ICGA). OCT reveals a dome-shaped hyperreflective lesion located between the retina and RPE [40]. The foveal thinning and EZ disruption are also observed and probably explain the progressive visual loss [41, 42].

AVMD progression is characterized by fragmentation and reabsorption of the vitelliform material [6]. Macular atrophy progressively replaces the vitelliform deposits at the advanced stages of the disease in most cases [42], but most patients retain reading vision throughout life [43, 44]. CNV may be complicated in few cases; 6 out of 51 patients developed CNV after a 6-year follow-up [45]. Anti-VEGF therapies have shown to be effective in the treatment of CNV associated with AVMD [46].

21.5 Genetic Aspects

21.5.1 BVMD

Currently, only genetic test for mutation analysis of the *BEST1* gene leads to confirmation of a clinical diagnosis of BVMD. Note that individuals with clinical findings of BVMD occasionally have a normal EOG, turning out to have a pathogenic variant of *BEST1* [47]. In case of atypical BVMD [3], genetic test for confirmation should be performed. Over 200 *BEST1* mutations with significant clinical heterogeneity require a thorough genetic analysis and clinical examinations to better understanding of genotype-phenotype correlations in BVMD. Most mutations of *BEST1* gene in BVMD and AVMD are missense mutations. Table 21.1 shows a list of missense mutations of *BEST1* gene in BVMD and AVMD.

Most genetic studies were performed in Western countries including the USA, England, Sweden, Denmark, Germany, the Netherlands, Italy, and France. *BEST1* mutations are extremely

Table 21.1 *BEST1* missense mutations in BVMD and AVMD

	Mutations a.a	Mutation nucleotide	Associated isease	Inheritance	Ethnicity	References
1	Thr2Ala	c.4A > G	BVMD	AD	Japanese	[48, 49]
			AVMD	AD	Iowa, USA	
2	Thr2Asn	c.5C > A	BVMD	AD	Chinese	[50]
3	Thr2Ile	c.5C > T	AVMD	AD	Iowa, USA	[49]
4	Ile3Asn	c.8 T > A	Atypical BVMD	AD	USA	[51]
5	Ile3Thr	c.8 T > C	BVMD	AD	Dutch	[52]
6	Thr4Ala	c.10A > G	BVMD	AD	French	[53]
7	Thr4Ile	c.11C > T	AVMD	AD	Chinese	[54, 49]
					Iowa, USA	
8	Tyr5His	c.13 T > C	AVMD	AD	Iowa, USA	[49]
9	Tyr5Term	c.15C > A	Multifocal	AD	French	[55]
			BVMD			
10	Thr6Ala	c.16A > G	BVMD	De novo	USA	[56]
11	Thr6Arg	c.17C > G	BVMD	AD	Iowa, USA	[57, 58]
				AD	USA or Swiss	
12	Thr6Lys	c.17C > A	AVMD	AD	Iowa, USA	49,
13	Thr6Pro	c.16A > C	BVMD	AD	Dutch	[59, 52, 60–62, 2]
			BVMD	AD	Dutch	
			Multifocal	AD	Dutch	
			BVMD			
			AVMD	AD	German	
			BVMD	AD	Dutch	
			BVMD	AD	Dutch	
14	Ser7Asn	c.20G > A	BVMD	AD	Japanese	[48]
15	Val9Ala	c.26 T > C	AVMD	AD	French	[53, 2]
					Swedish	
16	Val9Glu	c.26 T > A	BVMD	Unknown	Portuguese	[63]
17	Val9Leu	c.25G > C	BVMD	AD	USA	[64, 49]
			AVMD	AD	Iowa, USA	
18	Val9Met	c.25G > A	BVMD	AD	German	[65, 61, 66]
			BVMD	AD	German	
			BVMD	AD	German	
19	Ala10Thr	c.28G > A	BVMD	AD	German	[61, 66]
			BVMD	AD	German	
20	Ala10Val	c.29C > T	BVMD	AD	Dutch	[59, 62]
			BVMD	AD	Dutch	
21	Asn11Ile	c.32A > T	BVMD	AD	German	[67]
22	Arg13Cys	c.37C > T	AVMD	AD	Iowa, USA	[49]
23	Arg13His	c.38G > A	BVMD	AD	Chinese	[54, 68]
			BVMD	AD	USA	
24	Arg13Pro	c.38G > C	AVMD	AD	Iowa, USA	[49]
25	Gly15Arg	c.43G > C	BVMD	AD	Slovenian	[69]
26	Gly15Asp	c.44G > A	BVMD	AD	Italian	[53]
27	Ser16Phe	c.47C > T	BVMD	AD	Chinese	[70, 71]
			BVMD	AD	French	
28	Ser16Tyr	c.48C > A	BVMD	AD	Dutch	[59, 60]
			Multifocal	AD	Dutch	
			BVMD			

(continued)

Table 21.1 (continued)

	Mutations a.a	Mutation nucleotide	Associated isease	Inheritance	Ethnicity	References
29	Phe17Cys	c.50 T > G	BVMD	AD	French	[71, 58]
				AD	USA or Swiss	
30	Phe17Ser	c.50 T > C	AVMD	AD	Iowa, USA	[49]
31	Arg19Leu	c.56G > T	AVMD	AD	Iowa, USA	[49]
32	Leu20Val	c.58C > G	BVMD	AD	Danish	[12]
33	Leu21Val	c.61C > G	BVMD	AD	German	[61, 72]
			BVMD	AD	English, Canadian	
34	Trp24Cys	c.72G > T	BVMD	AD	USA or Swiss	[58, 66]
			BVMD	AD	German	
35	Arg25Gln	c.74G > A	BVMD	AD	German	[66]
36	Arg25Trp	c.73C > T	BVMD	AD	Japanese	[48, 53, 73, 58, 61]
			BVMD	AD	French	
			BVMD	AD	Italian	
			BVMD	AD	USA or Swiss	
			BVMD	AD	German	
37	Gly26Arg	c.76G > C	BVMD	AD	German	[67]
38	Ser27Arg	c.81C > G	BVMD	AD	German	[61]
39	Tyr29His	c.85 T > C	BVMD	AD	German	[67]
40	Lys30Arg	c.89A > G	BVMD	AD	USA or Swiss	[58]
41	Lys30Asn	c.90G > C	AVMD	AD	Iowa, USA	[49]
42	Glu35Lys	c.103G > A	BVMD	Unknown	Portuguese	[63]
43	Leu41Pro	c.122 T > C	BVMD	AD	German	[67]
44	Arg47Cys	c.139C > T	AVMD	AR	Iowa, USA	[49]
45	Arg47His	c.728C > T	BVMD	AD	Chinese	[70, 61]
			AVMD	AD	German	
46	Gln58Leu	c.173A > T	BVMD	AD	German	[65, 61]
			BVMD	AD	German	
47	Tyr72Asp	c.214 T > G	AVMD	AD	Iowa, USA	[49]
48	Ile73Asn	c.218 T > A	BVMD	AD	French	[71]
49	Ile73Phe	c.217A > T	BVMD	AD	USA	[64]
50	Leu75Phe	c.223C > T	BVMD	AD	Chinese	[50]
51	Ile76Asn	c.227 T > A	AVMD	AD	Iowa, USA	[49]
52	Ile76Val	c.226A > G	BVMD	AD	Iowa, USA	[49]
53	Phe80Leu	c.240C > A	BVMD	AD	Japanese	[48, 58]
			BVMD	AD	USA or Swiss	
54	Phe80Val	c.238 T > G	BVMD	AD	USA	[64]
55	Val81Met	c.241G > A	BVMD	AD	Japanese	[48, 49]
			BVMD	AD	Iowa, USA	
56	Leu82Val	c.244C > G	BVMD	AD	Danish	[12, 74, 52, 62]
			BVMD	AD	German	
			BVMD	AD	Dutch	
			BVMD	AD	Danish	
57	Phe84Val	c.250 T > G	AVMD	AD	Iowa, USA	[49]

(continued)

Table 21.1 (continued)

	Mutations a.a	Mutation nucleotide	Associated isease	Inheritance	Ethnicity	References
58	Tyr85His	c.253 T > C	BVMD	AD	Danish	[12, 74, 2]
			BVMD	AD	Danish	
			BVMD	AD	Swedish	
59	Val89Ala	c.266 T > C	BVMD	AD	Swedish	[75]
60	Thr91Ile	c.272C > T	BVMD	AD	French	[53, 58]
				AD	USA or Swiss	
61	Arg92Cys	c.274C > T	BVMD	AD	Italian,	[53, 62]
					French	
			BVMD	AD	Swedish	
62	Arg92Gly	c.274C > G	AVMD	AD	Italian	[53]
63	Arg92His	c.275G > A	BVMD	AD	Danish	[12, 74, 71]
			BVMD	AD	Danish	
			BVMD	AD	French	
64	Arg92Ser	c.274C > A	BVMD	AD	German	[65, 61]
			BVMD	AD	German	
65	Trp93Arg	c.277 T > C	AVMD	AD	Iowa, USA	[49]
66	Trp93Cys	c.279G > C	BVMD	AD	Swedish	[2]
67	Gln96Arg	c.287A > G	BVMD	AD	Danish	[12]
68	Gln96Glu	c.286C > G	AVMD	AD	Iowa, USA	[49]
69	Gln96His	c.288G > C	BVMD	AD	Dutch	[59, 62]
			BVMD	AD	Dutch	
70	Asn99Lys	c.297C > A	BVMD	AD	German	[61]
71	Asn99Tyr	c.295A > T	BVMD	AD	Iowa, USA	[49]
72	Leu100Arg	c.299 T > G	BVMD	AD	German	[67, 61]
			BVMD	AD	German	
73	Pro101Leu	c.302C > T	AVMD	AD	Iowa, USA	[49]
74	Pro101Thr	c.301C > A	BVMD	AD	USA or Swiss	[58]
75	Tryp102Arg	c.304 T > C	BVMD	AD	German	[67]
76	Asp104Glu	c.312C > A	BVMD	AD	Swedish	[2]
77	Asp104His	c.301G > C	BVMD	AD	German	[67]
78	Arg105Gly	c.313G > C	BVMD	AD	Slovenian	[69]
79	Phe113Leu	c.339C > G	BVMD	AD	Chinese	[76]
80	Arg130Ser	c.388C > A	BVMD	AD	USA	[64]
81	Asn133Lys	c.399C > G	BVMD	AD	USA or Swiss	[58]
82	Leu134Val	c.400C > G	BVMD	AD	Dutch	[59, 77, 60]
			BVMD	AD	French	
			Multifocal BVMD	AD	Dutch	
83	Gly135Ser	c.403G > A	BVMD	AD	USA or Swiss	[58, 62]
			BVMD	AD	Swedish	
84	Leu140Arg	c.419 T > G	BVMD	AD	USA or Swiss	[58]
85	Arg141His	c.422G > A	BVMD	AD	USA or Swiss	[58, 61]
				AD	German	
86	Arg141Ser	c.421C > A	BVMD	AR	Iowa, USA	[49]

(continued)

Table 21.1 (continued)

	Mutations a.a	Mutation nucleotide	Associated isease	Inheritance	Ethnicity	References
	Val143Phe	c.427G > T	AVMD	AD	Iowa, USA	[49]
87	Ser144Asn	c.431G > A	BVMD	AD	Chinese	[70, 50]
			BVMD	AD	Chinese	
88	Ser144Gly	c.430A > G	Multifocal	AD	French	[55]
			BVMD			
89	Ala195Val	c.584C > T	BVMD	AD	Japanese	[48, 59, 60, 67, 58]
			BVMD	AD	Dutch	
			Multifocal	AD	Dutch	
			BVMD	AD	German	
			BVMD	AD	USA or Swiss	
90	Ile201Thr	c.602 T > C	BVMD	AD	USA or Swiss	[58]
91	Ser209Asn	c.626G > A	BVMD	AD	English, Canadian	[61]
92	Leu211Thr	c.632 T > C	BVMD	AD	USA or Swiss	[58]
93	Arg218Cys	c.652C > T	BVMD	AD	Chinese	[54, 12, 67, 71, 50, 58, 62, 68]
			BVMD	AD	Danish	
			BVMD	AD	German	
			BVMD	AD	French	
			BVMD	AD	Chinese	
			BVMD	AD	USA or Swiss	
			BVMD	AD	Dutch	
			BVMD	AD	USA	
94	Arg218Gly	c.652C > G	BVMD	AD	Italian	[73]
95	Arg218His	c.653G > A	BVMD	AD	Japanese	[48, 59, 71, 58]
			BVMD	AD	Dutch	
			BVMD	AD	French	
			BVMD	AD	USA or Swiss	
96	Arg218Ser	c.652C > A	BVMD	AD	German	[67, 62]
			BVMD	AD	Swedish	
97	Arg218Gln	c.654 T > G	BVMD	AD	Dutch	[66]
98	Gln220Pro	c.659A > C	AVMD	AD	Iowa, USA	[49]
99	Cys221Phe	c.662G > T	BVMD	AD	Iowa, USA	[49]
100	Cys221Trp	c.663 T > G	BVMD	De novo	Italy	[78]
101	Gly222Glu	c.665G > A	BVMD	AD	Japanese	[48]
102	Gly222Val	c.665G > T	BVMD	AD	USA or Swiss	[58]
103	Leu224Met	c.670C > A	BVMD	AD	German	[61]
104	Leu224Pro	c.671 T > C	BVMD	AD	USA or Swiss	[58]
105	Tyr227Asn	c.679 T > A	BVMD	AD	Dutch	[59, 58, 66, 2]
			BVMD	AD	USA or Swiss	
			BVMD	AD	Dutch	
			BVMD	AD	Dutch	
106	Tyr227Cys	c.680A > G	BVMD	AD	USA or Swiss	[58, 66]
			BVMD	AD	Dutch	

(continued)

Table 21.1 (continued)

	Mutations a.a	Mutation nucleotide	Associated isease	Inheritance	Ethnicity	References
107	Tyr227Phe	c.680A > T	BVMD	AD	German	[79]
108	Trp229Gly	c.685 T > G	BVMD	AD	Chinese	[80]
109	Ile230Asn	c.689 T > A	AVMD	AD	Iowa, USA	[49]
110	Ile230Trh	c.689 T > C	BVMD	AD	French	[53]
111	Ser231Arg	c.693 T > G	BVMD	AD	German	[61]
112	Ser231Thr	c.692G > C	BVMD	AD	French	[77]
113	Ile232Asn	c.695 T > A	BVMD	AD	German	[79]
114	Pro233Ala	c.697C > G	BVMD	AD	Swedish	[81]
115	Pro233Gln	c.698C > A	BVMD	AD	French	[77]
116	Pro233Leu	c.698C > A	AVMD	AD	Iowa, USA	[49]
117	Leu234Pro	c.698C > T	BVMD	Unknown	USA	[18]
118	Val235Leu	c.703G > C	BVMD	AD	French	[71]
119	Val235Met	c.703G > A	BVMD	AD	Dutch	[66]
120	Thr237Arg	c.710C > G	BVMD	AD	German	[67, 61]
			BVMD	AD	German	
121	Thr237Ser	c.709A > T	BVMD	AD	German	[79]
122	Thr241Asn	c.722C > A	BVMD	AD	German	[67]
123	Val242Met	c.724G > A	BVMD	AD	Japanese	[48]
124	Ala243Thr	c.727G > A	BVMD	AD	Danish	[12, 61, 58]
			BVMD	AD	German	
			BVMD	AD	USA or Swiss	
125	Ala243Val	c.728C > T	BVMD	AD	Italian	[53, 67, 61]
			BVMD	AD	German	
			AVMD	AD	German	
126	Arg255Trp	c.763C > T	BVMD	AD	Chinese	[50]
127	Pro274Arg	c.821C > G	AVMD	AR	Iowa, USA	[49]
128	Phe276Leu	c.828C > G	BVMD	AD	USA or Swiss	[58]
129	Tyr284Cys	c.851A > G	BVMD	AD	Iowa, USA	[49]
130	Arg291Val	c.872C > T	BVMD	AD	Chinese	[54]
131	Glu292Lys	c.874G > A	BVMD	AD	Chinese	[70, 82]
			BVMD	AD	USA	
132	Gln293His	c.879G > C	BVMD	AD	Chinese	[54], 77]
			BVMD	AD	French	
133	Gln293Lys	c.877C > A	BVMD	AD	Dutch	[59, 62]
			BVMD	AD	Dutch	
134	Leu294Val	c.880C > G	BVMD	AD	German	[67]
135	Ile295Thr	c.884 T > C	BVMD	AD	German	[67, 83]
			BVMD	AD	Japanese	
136	Ile295Val	c.883A > G	BVMD	AD	Iowa, USA	[49]
137	Asn296His	c.886A > C	BVMD	AD	USA or Swiss	[58]
138	Asn296Lys	c.891C > A	Multifocal	AD	Dutch	[60]
			BVMD			
139	Asn296Ser	c.887A > G	BVMD	AD	Danish	[12, 71]
			BVMD	AD	French	
140	Pro297Ala	c.889C > G	BVMD	AD	USA or Swiss	[58, 66]
			BVMD	AD	Dutch	
141	Pro297Ser	c.889C > T	BVMD	AD	Iowa, USA	[49]

(continued)

Table 21.1 (continued)

	Mutations a.a	Mutation nucleotide	Associated isease	Inheritance	Ethnicity	References
142	Pro297Thr	c.889C > T	BVMD	AD	Chinese	[50]
143	Phe298Cys	c.893 T > G	BVMD	AD	USA	[64]
144	Phe298Ser	c.893 T > C	BVMD	AD	Dutch	[59, 60, 67]
			Multifocal	AD	Dutch	
			BVMD			
			BVMD	AD	German	
145	Phe298Val	c.892 T > G	BVMD	Unknown	English	[84]
146	Gly299Ala	c.896G > C	BVMD	AD	Dutch	[59, 52]
			BVMD	AD	Dutch	
147	Gly299Arg	c.895G > A	BVMD	AD	French	[77]
148	Gly299Glu	c.896G > A	BVMD	AD	Swedish	[2]
149	Glu300Asp	c.900G > C	BVMD	AD	Iowa, USA	[49, 58, 68]
			BVMD	AD	USA or Swiss	
			BVMD	AD	USA	
150	Glu300Lys	c.898G > A	BVMD	AD	Chinese	[70, 61, 58]
			BVMD	AD	German	
			BVMD	AD	USA or Swiss	
151	Asp301Asn	c.901G > A	BVMD	AD	German	[61]
152	Asp301Glu	c.903 T > G	BVMD	AD	German	[65, 67, 61, 68]
			BVMD	AD	German	
			BVMD	AD	German	
			BVMD	AD	USA	
153	Asp301Gly	c.902A > G	BVMD	AD	Chinese	[50, 54]
			BVMD	AD	Chinese	
154	Asp302Ala	c.905A > C	BVMD	AD	Danish	[12, 64, 59]
			BVMD	AD	USA	
			BVMD	AD	Dutch	
155	Asp302Asn	c.904G > A	BVMD	AD	Danish	[12]
156	Asp302Gly	c.905A > G	BVMD	AD	USA or Swiss	[58]
157	Asp302His	c.904G > C	BVMD	AD	French	[85]
158	Asp302Val	c.905A > T	BVMD	AD	USA or Swiss	[58]
159	Asp303Asn	c.907G > A	BVMD	AD	Italian	[86]
160	Asp303Glu	c.909 T > A	BVMD	AD	French	[85]
161	Asp303Gly	c.908A > G	AVMD	AD	Iowa, USA	[49]
162	Asp304Asn	c.910G > A	AVMD	AD	Iowa, USA	[49]
163	Asp304Gly	c.911A > G	BVMD	AD	Italian	[86]
164	Asp304Val	c.911A > T	BVMD	Unknown	Portuguese	[63]
165	Phe305Leu	c.915 T > A	BVMD	AD	Italian	[47]
166	Phe305Ser	c.914 T > C	BVMD	AD	Dutch	[66]
167	Phe305Tyr	c.914 T > A	AVMD	AD	Iowa, USA	[49]
168	Glu306Asp	c.918G > C	BVMD	AD	Japanese	[48, 58]
			BVMD	AD	USA or Swiss	
169	Glu306Gly	c.917A > G	BVMD	AD	USA or Swiss	[58]
170	Thr307Asp	c.920C > A	BVMD	AD	Chinese	[70]
171	Thr307Ala	c.919A > G	BVMD	AD	USA or Swiss	[58]

(continued)

Table 21.1 (continued)

	Mutations a.a	Mutation nucleotide	Associated isease	Inheritance	Ethnicity	References
172	Thr307Ile	c.902C > T	BVMD	AD	USA or Swiss	[58, 68]
				AD	USA	
173	Asn308Ser	c.923A > G	BVMD	AD	French	[85]
174	Trp309Arg	c.925 T > C	AVMD	AD	Iowa, USA	[49]
175	Ile310Thr	c.929 T > C	BVMD	AD	Germany	[61]
176	Val311Gly	c.932 T > G	BVMD	AD	Germany	[61]
177	Asp312Asn	c.934G > A	AVMD	AD	Germany	[61]
178	Asp312Glu	c.936C > A	BVMD	AD	Danish	[12, 74]
			BVMD	AD	Danish	
179	Gln316His	c.948G > T	AVMD	AR	Iowa, USA	[49]
180	Gln316Pro	c.947A > C	AVMD	AD	Iowa, USA	[49]
181	Pro346His	c.1037C > A	BVMD	AD	Japanese	[48]
182	Val492Ile	c.1474G > A	AVMD	AD	Iowa, USA	[49]
183	Glu557Lys	c.1669G > A	AVMD	AD	Iowa, USA	[49]

heterogenous, but several mutations have been frequently found (Thr6Pro, Arg25Trp, Arg218Cys, Tyr227Asn, Arg243Val, Ile295del, Gle300Asp, Asp301Glu, and Asp302Asn). Interestingly, these frequent mutations are ethnic specific (44.4% of Asp302Asn in Danish [12] and 36.8% of Arg25Trp in Italian [86]).

Currently, only limited reports are available in Asian genetic studies of BEST1 from Chinese [50, 54, 70, 76, 80, 87–89], Japanese [48, 83], and Korean [9]. The mutation spectrum of the *BEST1* gene in Asian patients of BVMD is differed from those in Western patients [88]. Six novel missense mutations (Thr2Asn, Leu75Phe, Ser144Asn, Arg255Trp, Pro297Thr, and Asp301Gly) and one previously reported mutation (Arg218Cys) were identified [50]. Three novel mutations Tyr4Ile [54], Ala291Val [54], and Phe113Leu [76] in BVMD were reported. Lin [80] reported two novel heterozygous mutations 304delAsp and Trp229Gly in Chinese BVMD patients. Liu [70] reported four previously reported mutations (Ser16Phe, Ser144Asn, Glu292Lys, and Glu300Lys) and two novel disease-causing mutations (Thr307Asp, Arg47His) in Chinese patients with BVMD.

In Japanese study [48], 22 patients including 16 probands from 16 families with BVMD were analyzed. All 16 probands exhibited characteristic BVMD fundus appearances, abnormal EOG, and normal ERG responses with the exception of one diabetic retinopathy proband. Genetic analysis identified 12 BEST1 variants in 13 probands (81%). Of these, ten variants (Tyr2Arg, Arg25Trp, Phe80Leu, Val81Met, Ala195Val, Arg218His, Gly222Glu, Val242Met, Asp304del, and Glu306Asp) have been previously reported in BVMD, while two variants (Ser7Asn and Pro346His) were novel disease-causing mutations.

In Korea, we report a BVMD patient (Fig. 21.1) carrying Asn296Lys mutation which is a causative mutation of multifocal BVMD in German patient [60]. Arg218Leu is a novel disease-causing mutation in BVMD (Fig. 21.2). These findings expand the spectrum of *BEST1* genetic variation in Asian and will be valuable for genetic counseling for patients with BVMD [88].

BVMD shows variable expressivity and incomplete penetrance at the clinical level. Disease-causing effect of *BEST1* mutations seems to be cumulative over time [79]. In genotype-phenotype relationship of Dutch study [59], median age of onset of visual symptoms was 33 years (range, 2–78). The cumulative risk of VA below 0.5 (20/40) was 50% at 55 years and 75% at 66 years. The cumulative risk of VA decline less than 0.3 (20/63) was 50% by age 66 years and 75% by age 74 years. Most patients (96%) had missense mutations; the Thr6Pro, Ala10Val, and Tyr227Asn mutations were most common. Visual decline was significantly faster in patients with an Ala10Val mutation than either the Thr6Pro or the Tyr227Asn mutation.

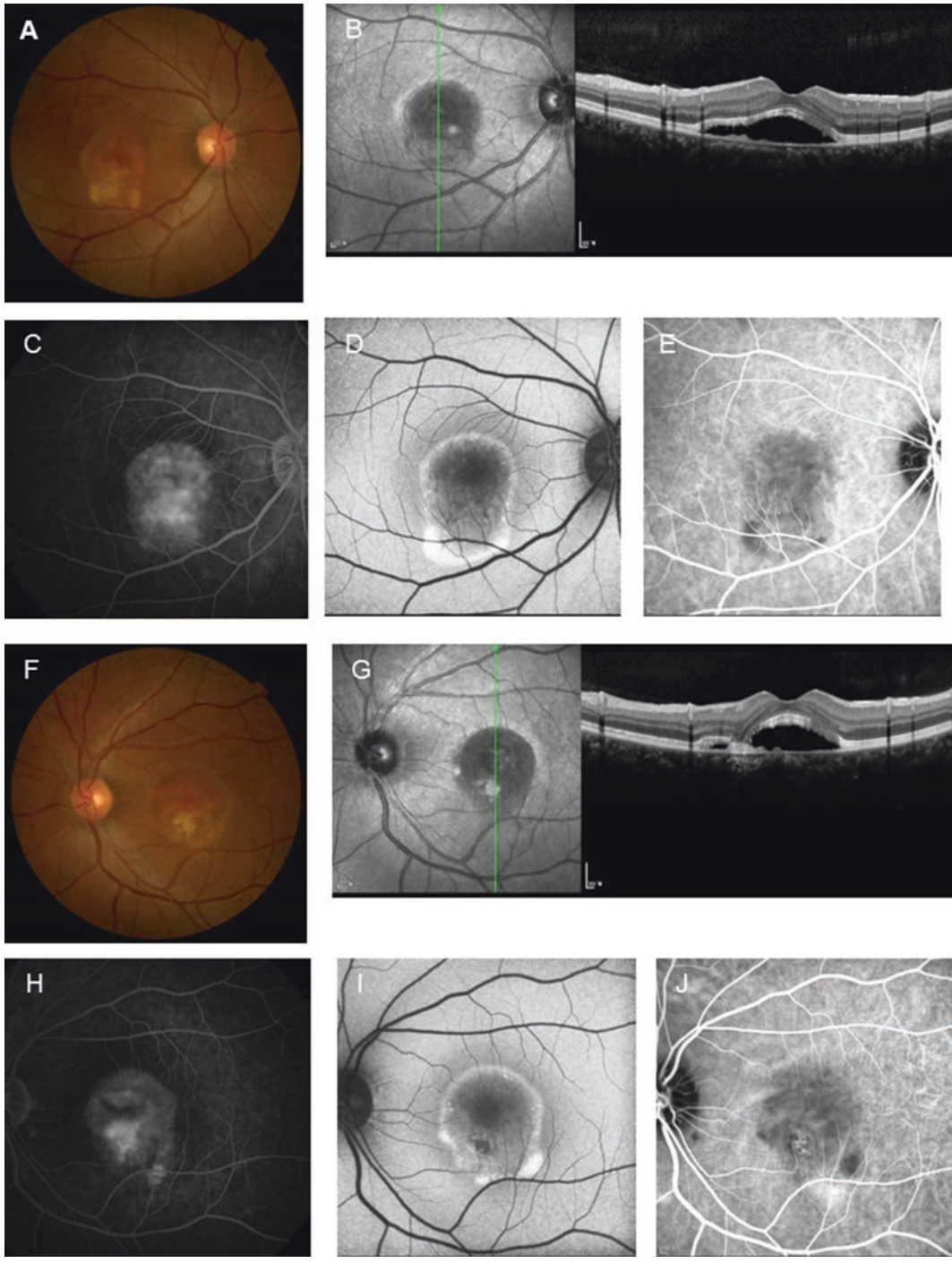

Fig. 21.1 Best vitelliform macular dystrophy (BVMD). A 32-year-old man carrying p.Asn296Lys mutation in the *BEST1* gene was incidentally found on routine fundus examination for a pilot license. The visual acuities (VA) were 20/20 in both eyes. (**a**, **f**) Bilateral BVMD of vitelliruptive stage shows scattered yellow-white vitelliform deposits. (**b**, **g**) Vertical optical coherent tomography (OCT) shows serous retinal detachment and hyperreflective vitelliform materials at RPE in both eyes. (**c**, **h**) Fluorescein angiography (FA) shows late pooling of fluorescein dye at the egg lesion. (**d**, **i**) Fundus autofluorescence (FAF) image of the vitelliruptive lesion shows increased autofluorescence at inferior part of ruptured vitelliform lesions and at the border of the serous retinal detachment. (**e**, **j**) Indocyanine green angiography (ICGA) shows active leakage spot in the left eye

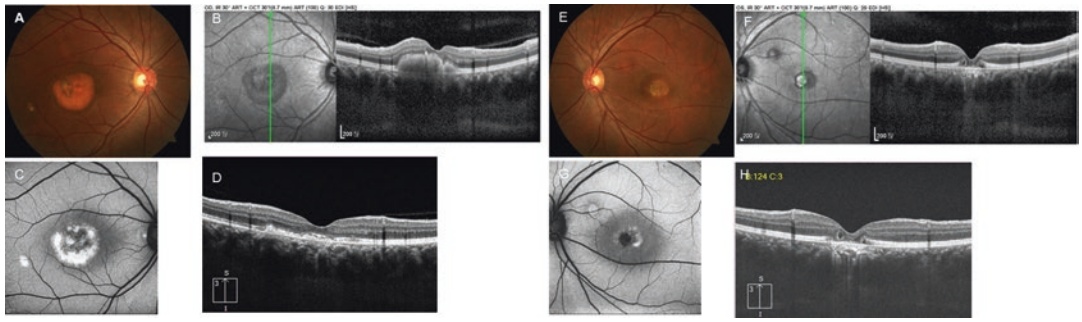

Fig. 21.2 Best vitelliform macular dystrophy (BVMD). A 39-year-old man carrying Arg218Leu mutation in the *BEST1* gene had multiple injections of anti-VEGF agents (ten for right eye and five for left eye) in both eyes. At initial visiting in our institute, vitelliform stage of right eye (**a**) reveals highly reflective subfoveal pillar without surrounding SRF (**b**). Small round vitelliform lesion with central cicatricial change was found in the left eye (**e**). OCT reveals marked RPE loss at the fovea (**f**). Six months later, FAF shows dispersed materials with hyperautofluorescent (**c**), and OCT reveals the disappearance of subfoveal pillar with a progression to vitelliruptive stage (**d**) in the right eye. FAF shows central hypoautofluorescent and surrounding hyperfluorescent lesions. OCT reveals that hypoautofluorescent lesion corresponds to the enlarged RPE loss (**h**)

In the recent Chinese study, despite typical macular appearance of BVMD, no clear genotype-phenotype correlation was observed [88]. In Asian BVMD cohort, genetic tests should be performed for the diagnosis with thorough clinical examinations to elucidate a genotype-phenotype correlation.

21.5.2 AVMD

In AVMD, several mutations in *BEST1* gene have been identified including p.Ala146Lys [90], p. Thr6Pro, p.Arg47His, P.Ala243Val, p.Asp312Asn [61], and p.Ile38Ser [9]. Table 21.1 includes the list of missense mutations in AVMD. In addition, AVMD is associated with mutations in *PRPH2* [91], *IMPG1* [92], *IMPG2* [93].

Age of onset is a major criterion to distinguish BVMD from AVMD [64]. Thus, systematic screening of *BEST1* and *PRPH2* has been suggested in BVMD and AVMD. *BEST1* screening should be recommended to patients with an age of onset less than 40 years, and *PRPH2* screening should be recommended to patients with an age of onset more than 40 years. For an onset between 30 and 40 years, *PRPH2* can be screened if no mutation has been detected in *BEST1*. In this screening approach, we found *PRPH2* mutation of p.Pro219_Pro221delinsPro in a 39-year-old female without *BEST1* mutation (Fig. 21.3).

21.6 Future Perspectives for Therapy

The development of gene and cell therapies is promising in various retinal diseases. Indeed, the results of clinical trials using iPSC-derived RPE cells in wet age-related macular degeneration [94] or AAV/RPE65 vectors in Leber's congenital amaurosis [95] were already reported. Therapeutic intervention of inherited retinal dystrophy should be primarily aimed at the restoration of normal gene (i.e., *BEST1* gene in BVMD and AVMD). However, until decade ago, this therapeutic goal was ideal but unachievable due to the lack of a proper biotechnology. Recent advances in genome editing technology using CRISPR system and gene delivery system are promising and harness the CRISPR-based genome editing for the therapeutic applications. Since its first therapeutic applications in retinal disease using wet AMD animal models [96, 97], in vivo genome editing using CRISPR-Cas9 enlarged its therapeutic applications both in genetic diseases harboring mutations [98, 99] and nongenetic degenerative diseases [96, 97, 100].

Conventional concept of gene therapy to deliver normal copy of *BEST1* gene into RPE would be effective in the treatment of VMD of haploinsufficiency phenotype, which is caused by *BEST1* mutations that exclusively result in a loss of sufficient wild-type protein. In addition,

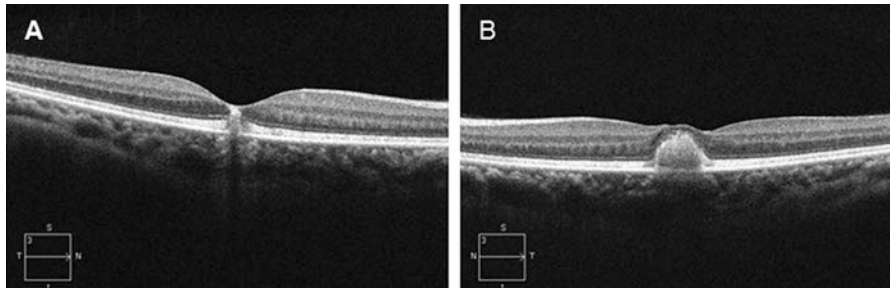

Fig. 21.3 Adult-onset vitelliform macular dystrophy (AVMD). A 39-year-old woman carrying Pro219_Pro221delinsPro in *PRPH2* gene suffered from dysmorphopsia of the right eye. OCT reveals subfoveal vitelliform lesion in the right eye (**a**) and left eye (**b**)

simple destruction of mutant proteins at the DNA level is achievable by genome editing of mutant *BEST1* allele using CRISPR-Cas9.

Currently, many BEST1 mutations cause VMD through dominant negative effect. In addition, over 200 mutations of *BEST1* gene, large amounts of *BEST1* mutations are missense mutations; thus, a precise base-editing using base-editors enables a literally complete recovery of normal gene [101, 102]. According to the recent advances in genome editing technology using CRISPR system, in vivo genome editing has emerged as a potential treatment strategy for inherited retinal dystrophies [103].

21.7 Summary

VMD is among the most common autosomal dominant macular dystrophy. Multimodal imaging with SD-OCT, FAF, FA, and ICGA is useful to the diagnosis of VMD. Genetic test is clinically important in the diagnosis of VMD because the clinical features of VMD can be similar to those of exudative AMD, CNV, or CSC. Future studies are needed to identify the prevalence with precise genetic mutations of *BEST1* in Asian VMD patients. This could provide a clear genotype-phenotype correlation in VMD. *In vitro* studies using RPE cells from patient-derived iPSC help to understand molecular biology of bestrophin-1 protein. Furthermore, in vivo genome editing using CRISPR-based base-editors might be a potential treatment strategy for the correction of missense mutations in VMD.

Compliance with Ethical Requirements Sung Wook Park, Chang ki Yoon, Dae Joong Ma, Un Chul Park, and Hyeong Gon Yu declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on institutional review board and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

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22 Adeno-Associated Virus (AAV)-Mediated Gene Therapy for Leber Hereditary Optic Neuropathy

Kunpeng Xie, Shuai Ming, Mingzhu Yang, Xuemin Jin, and Bo Lei

Abstract

Leber hereditary optic neuropathy (LHON) is the first clinically characterized maternally inherited mitochondrial disorder. Up to now, more than 30 pathogenic point mutations in mitochondrial DNA (mtDNA) coding for the respiratory chain subunits of complex I genes, which are highly susceptible to disrupted ATP production and oxidative stress, have been identified to cause LHON. The fundamental cell type affected in LHON is the retinal ganglion cells. Many researches facilitated the progress of animal models in vivo and cell culture in vitro that have been used to determine the effects of the genetic mutations upon the clinical phenotype and to explore potential novel therapies. More recently, clinical studies applying gene therapy have shown promising results in treating LHON. This article reviewed the efficacy and safety of recombinant adeno-associated virus 2 carrying ND4 (rAAV2-ND4) in clinical trials and its allotopic expression in the LHON patients with the G11778A mutation, which accounts for the majority of this vision-threatening disorder.

Keywords

Leber hereditary optic neuropathy · Allotopic expression · AAV2-ND4 · Gene therapy · Retinal ganglion cell

22.1 Introduction

Recently, gene therapy for monogenic inherited eye diseases has become a hot field. Promising results from different clinical trials have been reported for treating Leber hereditary optic neuropathy (LHON). LHON is a maternally inherited disease caused by point mutations in mitochondrial DNA (mtDNA), and it is considered as the most common mitochondrial disorder [1]. Three mtDNA mutations including m.3460G > A in MT-ND1, m.11778G > A in MT-ND4, and m.14484 T > C in MT-ND6 genes are found in almost 95% LHON patients [2]. They are considered as the primary mutations for causing LHON, and each mutation represents a significant risk of severe vision loss [3]. The prevalence of vision loss due to the three primary mutations in LHON is one in 31,000 in the Northern UK [4]. Other epidemiological studies reported a prevalence of one in 39,000 and one in 50,000 in the Netherlands and Finland, respectively [5, 6]. LHON is characterized by incomplete penetrance and is far more common in men. Usually, up to 50% of men and only 10% of women with the gene mutations present the

Author's Kunpeng Xie and Shuai Ming have been contributed equally to this chapter.

K. Xie · S. Ming · M. Yang · X. Jin · B. Lei (✉)
Henan Eye Institute, Henan Eye Hospital, Henan Provincial People's Hospital, Zhengzhou, China

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phenotype with typical symptoms of acute or subacute pain-free vision loss in one eye. Similar symptoms may appear in the other eye 8 weeks later averagely. The onset occurs typically in early adulthood, with little or no propensity to recover [7, 8]. Affected individuals are usually asymptomatic until they develop central vision loss in one eye.

Although great effort has been made, there is still no effective treatment for this devastating disease. Idebenone (a short-chain derivative of coenzyme Q10), EPI-743 (parabenzoquinone analogue of coenzyme Q10 and idebenone), and vitamin B12 are reported to be effective in some LHON patients, while the medication did not work in other patients [9]. Therefore, to find new regime for LHON, gene therapy has been explored in several studies due to the unique characteristics of the eye, including immune privilege, easy implementing of surgical intervention, and symmetrical disease progression [10, 11]. The present article reviewed the efficacy and safety of recombinant adeno-associated virus 2 (AAV2) carrying ND4 (rAAV2-ND4) in clinical trials and its allotopic expression in the LHON patients with G11778A mutation, which accounts for the majority of this vision-threatening disorder.

22.2 mtDNA Mutations and Construction of AAV-ND4 Vector

Point mutations in the ND1 (G3460A), ND4 (G11778A), and ND6 (T14484C) genes account for 95% of the LHON patients. Among them ND4 mutation (G11778A) identified in 70% patients impacts the most important complex I subunits of the mitochondrial respiratory chain and leads to reduction of ATP production and increase of reactive oxygen species (ROS) [3]. Complex I defect is the main cause of retinal ganglion cell (RGC) loss and optic atrophy [12]. Although gene therapy has gained great progress in the treatment for hereditary optic eye disease including LHON caused by mtDNA mutations, classical gene therapy is not applied to LHON yet for missing protein product. It is well known that mitochondrial structure includes two layers of membrane, which is the physical barrier for sustained gene expression following gene delivery into the mitochondrial matrix compartment [13]. Under these conditions, allotopic gene expression is employed to treat disease caused by mtDNA mutations. Adeno-associated viral vector serotype 2 (AAV2) is one of the most commonly used vectors for allotropic gene expression. As a therapeutic strategy, it has been developed to deliver the MT-ND4 gene construction to compensate for the m.11778G > A mutation [14].

AAV is a small, non-enveloped, icosahedral virus that contains a linear single-stranded DNA genome of 5 kb [15]. The AAV genome includes two frames: rep is required for DNA replication, and cap encodes all the three structural proteins which form the icosahedral capsid and an essential protein for capsid assembled within the nucleolus [16]. The AAV genome is flanked by two 145-bp-long palindromic inverted terminal repeats (ITRs) which form hairpin-loop secondary structures at the strand termini. Rep and cap are replaced by the exogenous DNA, which is provided together with the adenoviral helper regarding the AAV productive infection in the packaging cells [17]. AAV vectors are capable to infect quiescent cells and mediate long-term expression of transgenes. Various serotypes exhibit tropisms for different subsets of retinal cells. Among all of the AAV vectors, AAV2 is the most commonly used AAV serotype and has been applied to several genetic and degenerative eye diseases including LHON. In a series of laboratory experiments and clinic trials, ND4 subunit of complex I is packaged into AAV and then injected into the vitreous of rodent, nonhuman primate, and ex vivo human eye. AAV2-ND4 is modified with mitochondrial targeting signals and 3′ UTRs from nuclear genes whose mRNA has been found to localize to the mitochondrial surface, which results in targeting of the hybrid mRNA to the mitochondrial surface and in the increase in the amount of protein fully imported in mitochondria [18].

In a study by Guy and colleagues, the ND4 subunit of complex I is recoded and imported

into the mitochondria from the cytoplasm by adding a targeting sequence derived from the P1 isoform of the subunit c of ATP synthase [19]. Afterward, the nuclei encode ND4 with the appended subunit c of ATP synthase targeting sequences as P1ND4v2, which is inserted into a self-complementary adeno-associated virus vector (scAAV2) (Y444, 500, 730F) and named scAAV2-P1ND4v2 [20]. In another study by Yang et al., an AAV2-ND4 vector is constructed [21]. The DNA sequence is synthesized as two parts: the mitochondrial positioning signal (MTS) of the COX10 plus the coding sequence of ND4, with 5′ end carrying a KpnI site and 3′ end carrying a SalI site, and COX10 3′ UTR, with 5′ end carrying a SalI site and 3′ end carrying a BamHI site [14, 22]. Then the two sequences are inserted into the plasmid pAAV-2neo carrier successively [23].

22.3 The Safety of AAV-ND4-Mediated Gene Therapy for LHON

Safety is the most important issue to be addressed in gene therapy. In the study by Wan et al., patients with negative anti-AAV2 antibody were recruited for clinical trials. Oral prednisone was administered 1 week before and for 2 months thereafter to avoid a potentially severe immune response induced by gene therapy [23]. During the follow-up period of 36 months, there was no adverse event such as cataracts, retinal detachment, and endophthalmitis, or other ocular tissue damage in any of the subjects. Also, systemic examinations did not reveal any abnormal changes. None of the patients have remarkable change in the concentrations of serum AAV2, ND4, and IFN-γ. In the clinical trials carried out by Guy and colleagues, there were no serious safety concerns associated with allotopic gene therapy in 14 participants treated with low- and medium-dose vector. The primary adverse event was anterior uveitis related to the transgene. Fortunately, the inflammation response was mild, asymptomatic, and transient and required no treatment. Anterior uveitis was not reported in the study of Li's group [21, 23]. The discrepancy of occurrence of transient uveitis between the two studies remains unknown. One possible interpretation may be that one study used prednisone while the other did not. However, involvement of neutralizing antibodies (NAbs) against AAV2 can't be excluded, because one of the patients in Feuer's study showed elevation of NAbs [20]. NAbs to AAV2 in serum and aqueous humor were evaluated in the study by Guy et al. Elevated serum NAbs are found in 3 of all 14 cases. Though four patients with the most improvement in visual acuity after treatment had the highest levels of serum NAbs, they had similar low levels of aqueous humor NAbs, which suggested high level of serum NAbs was not associated with prognosis of visual acuity.

22.4 AAV-ND4-Mediated Gene Therapy Improved Patients' Visual Acuity

In all clinical trials reported by Yang and colleagues, the change in best-corrected visual acuity (BCVA) was the primary endpoint for LHON gene therapy [21]. Improvement of visual acuity was defined as an improvement of 0.3 or more with logMAR. This definition minimized the influence of subjective factors and changes in vision greater than or equal to 0.3 logMAR during the 36-month follow-up period. Five patients achieved vision improvement in the injected eyes between 3 and 6 months after gene therapy. Interestingly, vision improvement was also observed between 3 and 12 months in the contralateral un-injected eyes. Thus vision improvement in the injected eyes had a time window, while vision recovery observed in the fellow eyes happened randomly. There were four patients with no significant improvement in visual acuity 36 months after gene therapy [21].

There was no difference in vision improvement between patients with a disease duration less than 2 years and those more than 2 years. Though there was no difference with regard to the primary endpoint, gene therapy in the injected eye displayed an overall protection against the

further vision loss. No patient demonstrated a more serious decline in visual acuity from the baseline.

In the study reported by Guy and colleagues, vision improvement was defined as three lines or more [19]. One of the six patients with duration of vision loss longer than 12 months had visual improvement, while four of six patients with duration of vision loss within 1 year had visual improvement. Furthermore, the results indicated that vision recovery may be associated with the treatment dosages and duration of disease. In those participants with chronic vision loss more than 12 months and marked loss of RNFL, low dose of gene transfer presented no effect on visual acuity. However, when given a medium dose, one of the participants with marked loss of the RNFL showed an increase in visual acuity equivalent to three lines on the ETDRS chart. In those participants with acute visual loss who also had normal RNFL, an injection of low dose increased the acuity by three lines on the ETDRS chart. Vision improvements in the treated eyes were usually evident within 7–30 days after injection, as quickly as that reported in the Chinese cohort. Vision of these patients may improve further with longer follow-up period. The rapid and persistent improvement may result from the rapid and persistent expression of the transgene in the eyes, which was demonstrated in rodent studies [24]. Animal studies may provide important information; however, care must be taken in extrapolating the results in rodent to humans, particularly under pathological conditions.

It should be noted that there are two unexpected phenomena observed in the 3-year LHON gene therapy follow-up. Firstly, though visual acuity improvement of patients was above the baseline after treatment, some patients presented BCVA fluctuations during the 3-year follow-up. Visual acuity fluctuations may relate to stability of protein expression after ND4 transfection, other persistent disease factors, and/or patient-specific factors. Secondly, visual acuity of the contralateral eye appeared to be improved in some patients. Improvements of the un-injected eyes never occur before the improvements of the injected eyes, and it occurred within 6 months after injection, suggesting that the vision recovery of the contralateral eyes may be a result from gene therapy.

The mechanism by which gene therapy affected the contralateral eye was investigated [25]. In animal experiments, a fluorescent gold tracer was used to explore the link between the two eyes. The results demonstrated a physical communication between the two eyes via the optic chiasm. Studies by Luo et al. [26] showed that a few regenerating nerve axons crossed the optic chiasm into the contralateral optic nerve and grew toward the contralateral retina. These data suggested the possibility of direct communication between the optic nerves, which may contribute to the visual acuity improvement of the contralateral eyes.

22.5 AAV-ND4-Mediated Gene Therapy Prevented the Loss of the RNFL

Previous studies have shown that RNFL thickness continuously declines in LHON patients [27, 28]. To evaluate the effect of gene therapy on RNFL thickness, RNFL thickness was examined in the Chinese subjects. As expected, the RNFL thickness in the un-injected eyes decreased even after the gene therapy. However, RNFL thickness in the injected eyes did not change, suggesting that loss of RNFL terminated. These results suggested that gene therapy provided RNFL protection for the injected eyes, but did not have protective effect on the un-injected eyes 24 months after gene therapy. Studies reported by Guy et al. [19] showed that the temporal RNFL of treated patients followed up for 12 months was not damaged by allotopic ND4 gene therapy. One-year posttreatment values were similar to those obtained before treatment. In contrast, the thickness of the temporal RNFL of the fellow eyes continued to decrease. OCT data from both groups showed that allotopic ND4 was not harmful to visual function and did not damage the temporal RNFL subserving macular fibers targeted by an intravitreal AAV2 injection. These

results might pave the way for injection of high-dose cohorts in the following trials.

22.6 AAV-ND4 Decreased the PERG Amplitudes

In the study by Wan et al., VEP results showed that the latency period of the P100 wave of the injected eyes in some patients was shortened (a P100 wave <105 ms is normal) [23]. At 6 months after intravitreal injection, the P100 amplitudes of the injected eyes were increased, but VEP results were unstable. In the study by Guy et al., results suggested that PERG amplitudes worsened more in the treated eyes than the fellow eyes (P¼ 0.009 exchangeable; P ¼ 0.011 autoregressive) by approximately 0.05 mV [19]. Besides, in Guy's study, they inferred that the drop in PERG amplitudes seemed to be related to the injection, because the gene expression from the vector could not be initiated immediately.

We should consider that these results and analysis have certain limitations, including the possible introduction of biases resulting from the study population race and sample size, testing time, physician's behavioral differences, and other uncontrolled variables. In addition, the sample size was small.

22.7 Perspectives

LHON is a neurodegenerative mitochondrial disease causing devastating visual loss. It is obligatory to develop and validate new treatment strategies. In recent years, clinical trials involving gene therapy to treat the disease are emerging. Significant advances have been made in the use of viral vectors for ocular gene therapy. Results from the clinical trials of the AAV2-ND4 vector-mediated gene therapy are encouraging. Furthermore, as an alternative to using ND4 in viral vector, NDI1, a yeast nuclear gene which encodes a complex I equivalent, has been used in murine and rat models, demonstrating its efficacy in protecting LHON from the rotenone damage. This yeast gene would treat all mutations associated with complex I disease by replacing the entire complex I, not just the affected ND4 subunit [29, 30]. All of these developments bring much optimism about the future of LHON treatment. However, the efficacy, adverse effects, and duration of treatment benefit have yet to be determined by long-term follow-up.

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Stargardt Disease in Asian Population

23

Xiao Liu, Yu (Yokokawa) Fujinami, Lizhu Yang, Gavin Arno, Kaoru Fujinami

Abstract

Stargardt disease 1 (STGD1; MIM 248200) is the most prevalent inherited macular dystrophy, which is an autosomal recessive condition caused by pathogenic variants in the ABCA4 gene (ATP-binding cassette subfamily A member 4; MIM 601691). Clinical and molecular genetic investigations of STGD1/ABCA4 have been intensively performed over the last 10 years, and understanding the underlying pathophysiology promotes ongoing and planned human clinical therapeutic trials. We herein describe the phenotypic and genotypic characteristics of the disease, pathogenesis, therapeutic approaches, and recent findings in Asian population.

Keywords

Stargardt disease · ABCA4 · Macular dystrophy · Cone–rod dystrophy · Retinitis pigmentosa · Asian · Genetics

East Asian Inherited Retinal Disease Society (EAIRDs)

X. Liu
Laboratory of Visual Physiology, Division of Vision Research, National Institute of Sensory Organs, National Institute of Sensory Organs, Tokyo Medical Centre, Tokyo, Japan

Keio University, Tokyo, Japan

Third Military Medical University, Chongqing, China

Y. (Yokokawa) Fujinami
Laboratory of Visual Physiology, Division of Vision Research, National Institute of Sensory Organs, National Institute of Sensory Organs, Tokyo Medical Centre, Tokyo, Japan

Keio University, Tokyo, Japan

Yokokawa Clinic, Osaka, Japan

L. Yang
Laboratory of Visual Physiology, Division of Vision Research, National Institute of Sensory Organs, National Institute of Sensory Organs, Tokyo Medical Centre, Tokyo, Japan

Keio University, Tokyo, Japan

Peking Union Medical College Hospital, Beijing, China

G. Arno
Laboratory of Visual Physiology, Division of Vision Research, National Institute of Sensory Organs, National Institute of Sensory Organs, Tokyo Medical Centre, Tokyo, Japan

UCL Institute of Ophthalmology, London, UK

Moorfields Eye Hospital, London, UK

K. Fujinami (✉)
Laboratory of Visual Physiology, Division of Vision Research, National Institute of Sensory Organs, National Institute of Sensory Organs, Tokyo Medical Centre, Tokyo, Japan

Keio University, Tokyo, Japan

UCL Institute of Ophthalmology, London, UK

Moorfields Eye Hospital, London, UK
e-mail: k.fujinami@ucl.ac.uk

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23.1 Introduction

Stargardt disease or Stargardt macular dystrophy (STGD1: Online Mendelian Inheritance in Man identifier; 248200), first described by Karl Stargardt in 1909, is one of the most common macular dystrophy [1–5]. The prevalence of STGD1 has been estimated to be 10–12.5 per 100,000 [2, 6]. Most cases present with central visual loss, which often begins within the first/second decades of life, and there is typically macular atrophy with yellow-white flecks at the level of the retinal pigment epithelium (RPE) of the posterior pole on ophthalmoscopy (Fig. 23.1). However, there are various manifestations resulting in a large spectrum of clinical presentations, onset, progression, psychophysical and electrophysiological findings, and variable prognosis [4, 5, 7–17].

In 1997, causative mutations in the ABCA4 (ATP-binding cassette subfamily A member 4: Online Mendelian Inheritance in Man identifier; 601691) gene was firstly reported in patients with autosomal recessive Stargardt macular dystrophy [18]. The carrier frequency for a mutation in *ABCA4* may be as high as 1:20, and the true prevalence of retinopathy caused by disease-causing *ABCA4* variants is likely much higher than that of STGD1 [6, 19]. The vast allelic heterogeneity of *ABCA4* is also clearly demonstrated by the number of reported sequence variations (>1000) in the ABCA4 gene [6, 8, 19–26].

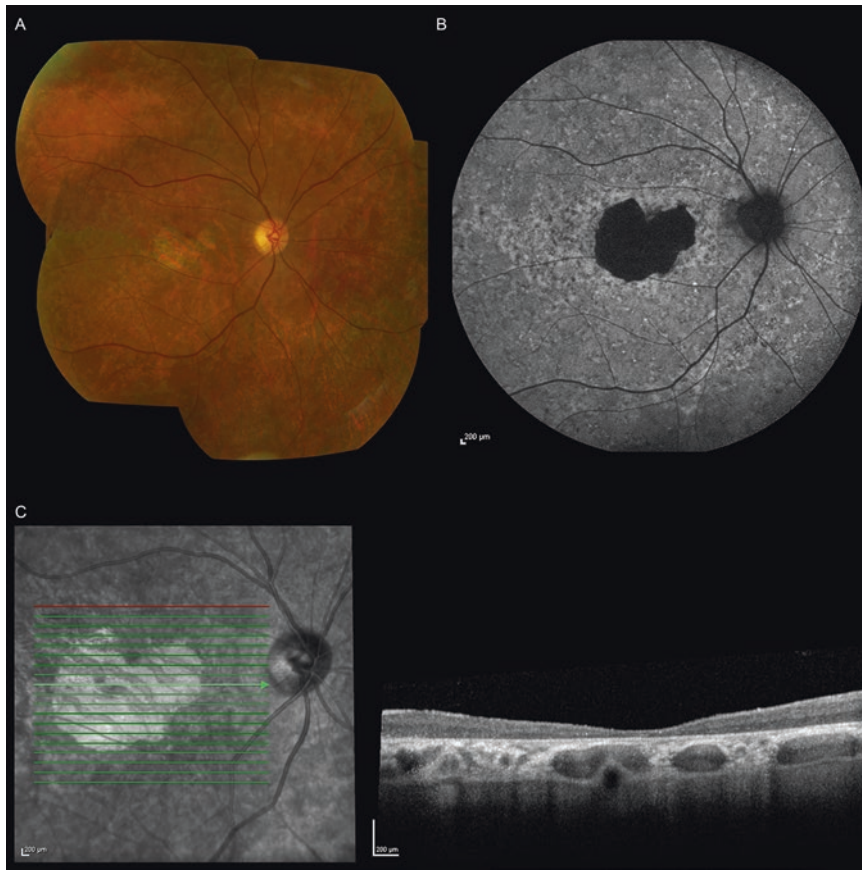

Fig. 23.1 Typical findings of Stargardt disease (STGD1) Fundus photography shows macular atrophy with yellow-white flecks at the level of the retinal pigment epithelium (RPE) at the posterior pole (**a**). Autofluorescence (AF) imaging reveals the area of low density at the macula and multiple foci of abnormal AF (**b**). Spectral-domain optical coherent tomography (SD-OCT) demonstrated the thinned sensory retina and RPE at the macula with multiple hyper refractive lesions corresponding to the flecks (**c**)

Clinical and molecular genetic investigations of STGD1/*ABCA4* have been intensively performed over the last 10 years, and understanding the underlying pathophysiology promotes ongoing and planned human clinical therapeutic trials [2, 27–29]. However, most of these researches are focusing on the Caucasian patients, and characteristics of Asian or African patients are still unknown [30–34]. We herein describe the phenotypic and genotypic characteristics of the disease, pathogenesis, therapeutic approaches, and recent findings in Asian population.

23.2 Molecular Genetics

The ABCA4 gene (cytogenetic location, 1p22.1; genomic coordinates (GRCh38), 1:93,992,836-94,121,148) is a large and highly polymorphic gene. The estimated size of *ABCA4* is 6,819 bp encoding a 2,273-amino acid protein, including 50 exons [18, 35]. Over 1000 variants in the ABCA4 gene are associated with macular dystrophy, cone dystrophy, cone–rod and 'rod–cone' dystrophy [4, 5, 7, 9–12, 14–17, 20]. Recently, the phenotype caused by ABCA4 gene aberration is described as 'ABCA4-associated retinal disorder.' [6, 14] This allelic heterogeneity makes it challenging to establish genotype–phenotype correlations [11]. Generally, null/deleterious variants are associated with earlier-onset disease with more severe phenotype and missense variants with later-onset disease with milder phenotype; meanwhile, certain missense variants can have severe functional effects similar to nulls (e.g. p.Leu541Pro/p.Ala1038Val (complex), p.Glu1022Lys, p.Cys1490Tyr, p.Glu1087Lys, p.Thr1526Met, p.Arg1640Trp, and p.Cys2150Tyr) [11, 17, 20, 36]. The interaction between the variants (including disease-causing and benign variants) may also affect the functional effects [37]. Certain missense variants including p.Arg2030Gln are commonly observed in the mildest *ABCA4*-associated phenotype, foveal-sparing Stargardt disease (FS-STGD1) [12, 16].

23.3 Disease Mechanism

ABCA4, formerly described as ABCR, is a member of the ABC transporter gene superfamily, encoding the retinal specific transmembrane protein, a member of the ATP-binding cassette transporter superfamily [38, 39]. ABCA4 is localised to the rim of the rod–cone outer segment discs and involved in the active transport of retinoids from photoreceptor to RPE in the retinoid cycle [38–41]. There are two transmembrane domains (TMD), two glycosylated extracellular domains (ECD), and two nucleotide-binding domains (NBD) (Fig. 23.2) [39].

The retinoid cycle consists of enzyme-catalysed reactions converting all-trans retinal, generated with photobleaching of rhodopsin/cone opsin, back to 11-cis retinal [38–40, 42, 43]. All-trans retinal is released from the light-activated rhodopsin/cone opsin into the rod–cone outer segments to form a complex with phosphatidylethanolamine (PE) resulting in N-retinylidene-phosphatidylethanolamine (N-ret-PE); then this complex is actively transported to the disc surface by ABCA4 (Fig. 23.2).

Failure of transport due to ABCA4 dysfunction/mislocalisation leads to inefficient removal of N-ret-PE from photoreceptor outer segments and results in an accumulation of bisretinoid compounds in outer segment discs, and ultimately in toxic levels of bisretinoid A2PE in photoreceptor membranes [23, 39, 41, 44]. A2PE is hydrolysed to form the highly toxic metabolite N-retinylidene-N-retinyl-ethanolamine (A2E), which accumulates as a major component of lipofuscin in RPE cells and ultimately causes RPE dysfunction and death with subsequent photoreceptor dysfunction/loss (Fig. 23.2) [42, 45]. The previous studies of STGD1 mouse model (ABCA4 knockout) support the aforementioned pathogenesis, although there are limitations such as lack of a macula in mice and the mild phenotype in mouse model showing a later-onset disease with slower degeneration than that of typical human patients with STGD1 [40, 46].

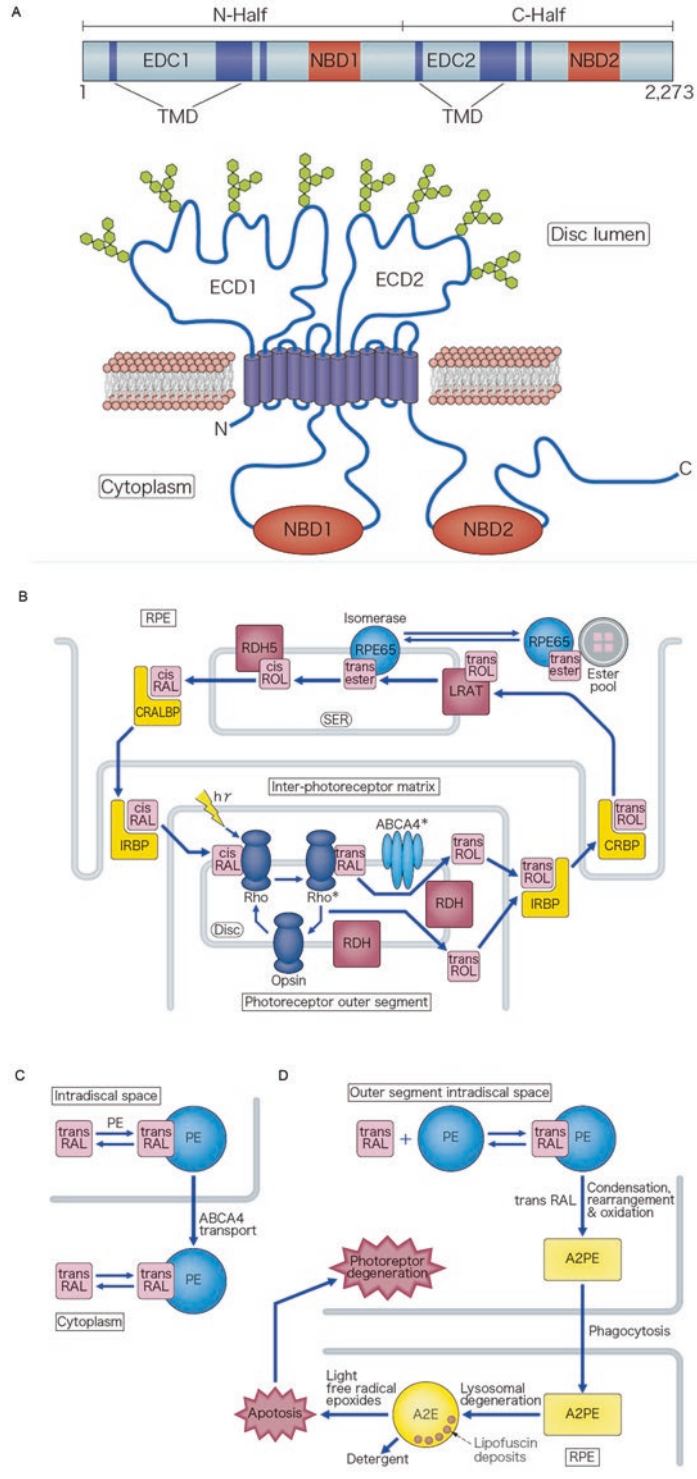

Fig. 23.2 A schematic of ABCA4 protein structure, retinoid cycle, transport of all-trans retinal, and disease mechanism The ABCA4 gene transcribes a large retina-specific ABCA4 protein with two transmembrane domains (TMD), two glycosylated extracellular domains (ECD), and two nucleotide-binding domains (NBD) (**a**). The retinoid cycle consists of enzyme-catalysed reactions converting all-trans retinal, generated with photobleaching of rhodopsin/cone opsin, back to 11-cis retinal (**b**). All-trans retinal is released from the light-activated rhodopsin/cone opsin into the rod–cone outer segments to form a complex with phosphatidylethanolamine (PE) resulting in N-retinylidene-phosphatidylethanolamine (N-ret-PE); then this complex is actively transported to the disc surface by ABCA4 (**c**). Failure of this transport results in accelerated deposition of a major lipofuscin fluorophore, N-retinylidene-N-retinylethanolamine (A2E), in the RPE, which causes RPE dysfunction and cell death, with subsequent photoreceptor cell loss overtime

23.4 Clinical Characteristics

Patients with STGD1 commonly present with progressive bilateral central vision loss. The onset is often the first/second decades of life but variable [4, 5, 12, 14, 47]. The onset relates to the disease severity; an earlier-onset disease is associated with more deleterious variants compared with adult-onset disease (more frequently, missense variants) [14].

Comprehensive investigations are crucial for clinical diagnoses, including fundus photography, AF imaging, spectral-domain optical coherence tomography (SD-OCT), and electrophysiological findings (Figs. 23.3, 23.4, 23.4, 23.5, and 23.6, Tables 23.1, 23.2, and 23.3). Clinical classifications are useful to assess the disease severity and associate with genotype group classification (Table 23.4).

At an early stage, ophthalmoscopy can reveal a normal or minimal retinal abnormalities, including foveal reflex abnormality and RPE disturbance, with or without vision loss [14]. Retinal imaging with AF, SD-OCT, and electrophysiological assessment (including pattern, full-field, and multifocal electroretinograms; PERG, FFERG, mfERG) is useful for the diagnosis [2, 14, 15, 48]. It is of note that paediatric patients with STGD1 may not have retinal flecks on fundoscopy or AF at the early stage and develop them associated with increasing macular atrophy overtime (Fig. 23.4). In very early childhood-onset disease with relatively preserved vision, macular atrophy involves the parafovea and spares the foveola, and these changes are predated by symmetrical yellowish-white fine dots at the central macula in some cases [14, 15, 48].

Electrophysiological assessment is helpful in confirming the diagnosis of STGD1 and in providing better-informed advice on prognosis. A classification of three functional phenotypes based on electrophysiological findings is well-established: Group 1, severe PERG abnormality (macular dysfunction) with normal FFERGs; Group 2, severe PERG abnormality with additional generalised cone dysfunction of FFERGs; and Group 3, severe PERG abnormality with additional generalised cone and rod dysfunction of FFERGs (Fig. 23.6) [8]. A longitudinal study demonstrated prognostic implications and that they do not reflect stages of disease: Group 1, the best prognosis; Group 2, intermediate variable prognosis; and Group 3, the worst prognosis [4]. All patients with initial rod ERG involvement demonstrated clinically significant electrophysiological deterioration; only 20% of patients with normal FFERGs showed clinically significant progression [4]. Such data are supported by association with genotype grouping and are also relevant in the design, patient selection, and monitoring of potential therapeutic interventions [4].

STGD1 with a later age of onset has been increasingly been recognised recently. Patients with late-onset STGD1 often have foveal-sparing phenotype (FS-STGD1) (Fig. 23.7) [10, 12, 49, 50]. Patients with FS-STGD1 frequently have preserved visual acuity and relatively maintained foveal structure and function [12]. Interestingly, SD-OCT often demonstrates the outer retinal tabulation at the edge of atrophy, which suggests the primal damage of this phenotype is RPE/choroid. On the other hand, patients with foveal atrophy show the sensory retinal atrophy at the fovea at the early stage. Therefore, the presence of two distinct phenotypes (non-FS-STGD1 and FS-STGD1) suggests that there may be more than one disease mechanism in *ABCA4*-associated retinal disorder [12]. The fact that the distribution of disease-causing variants between the two phenotypes is different can support this hypothesis [12].

23.5 Asian Population

The number of studies focusing on Asian population is relatively small; however several studies have been performed in Asian patients with STGD1/*ABCA4*-associated retinal disorder. The prevalent variants of recent reports from Chinese, Japanese, Korean, Indian, and South Asian have been summarised in Table 23.6.

Jing et al. reported the disease-causing variants in a Chinese cohort of 161 unrelated patients with STGD1 (96 patients) and cone–rod dystro-

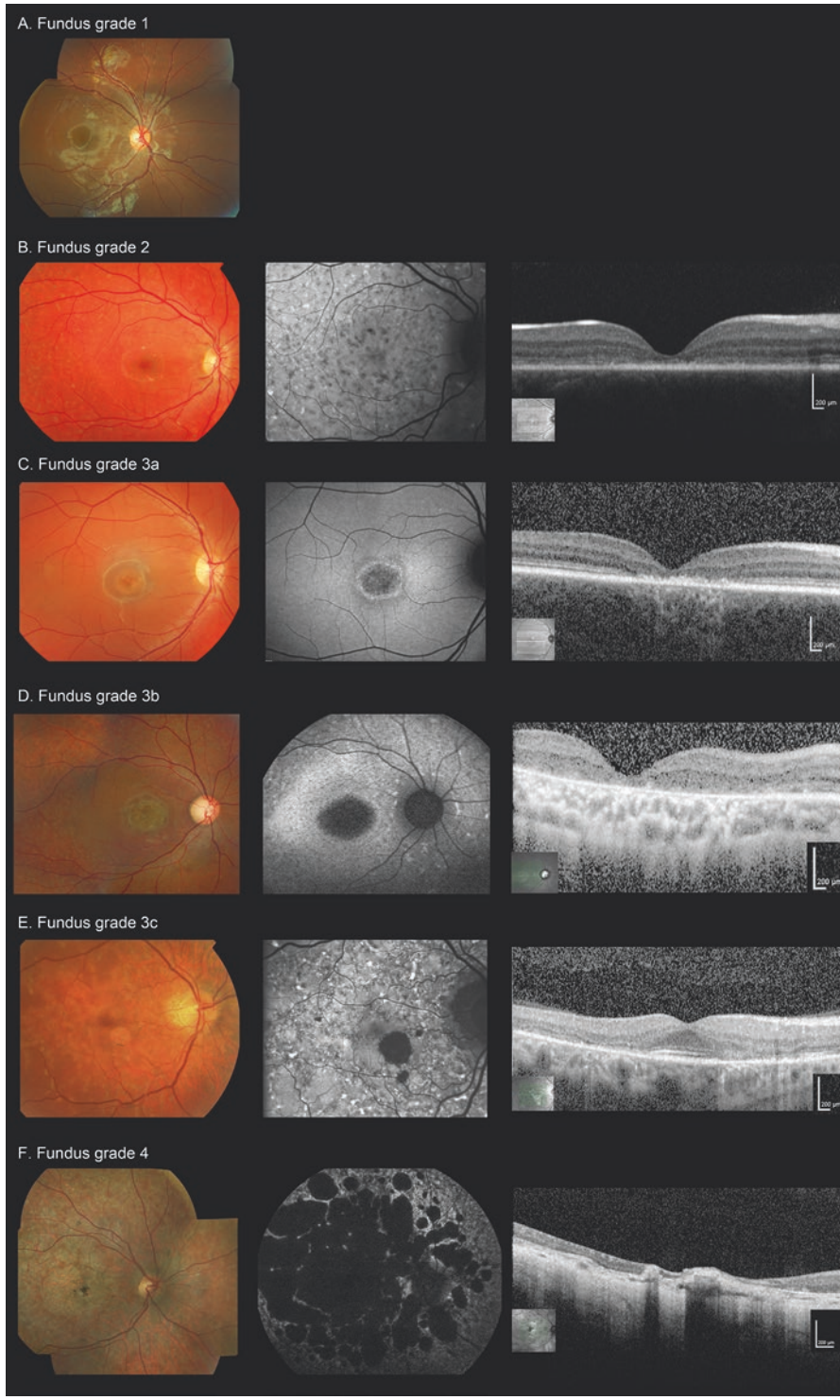

Fig. 23.3 Classification for fundus appearance in STGD1. Fundus classification is performed based on the presence of macular atrophy, flecks, foveal sparing, and peripheral atrophy. Detailed descriptions are presented in Table 23.1. (Fujinami et al. Clinical and Molecular Characteristics of Childhood-Onset Stargardt Disease. Ophthalmology 2015)

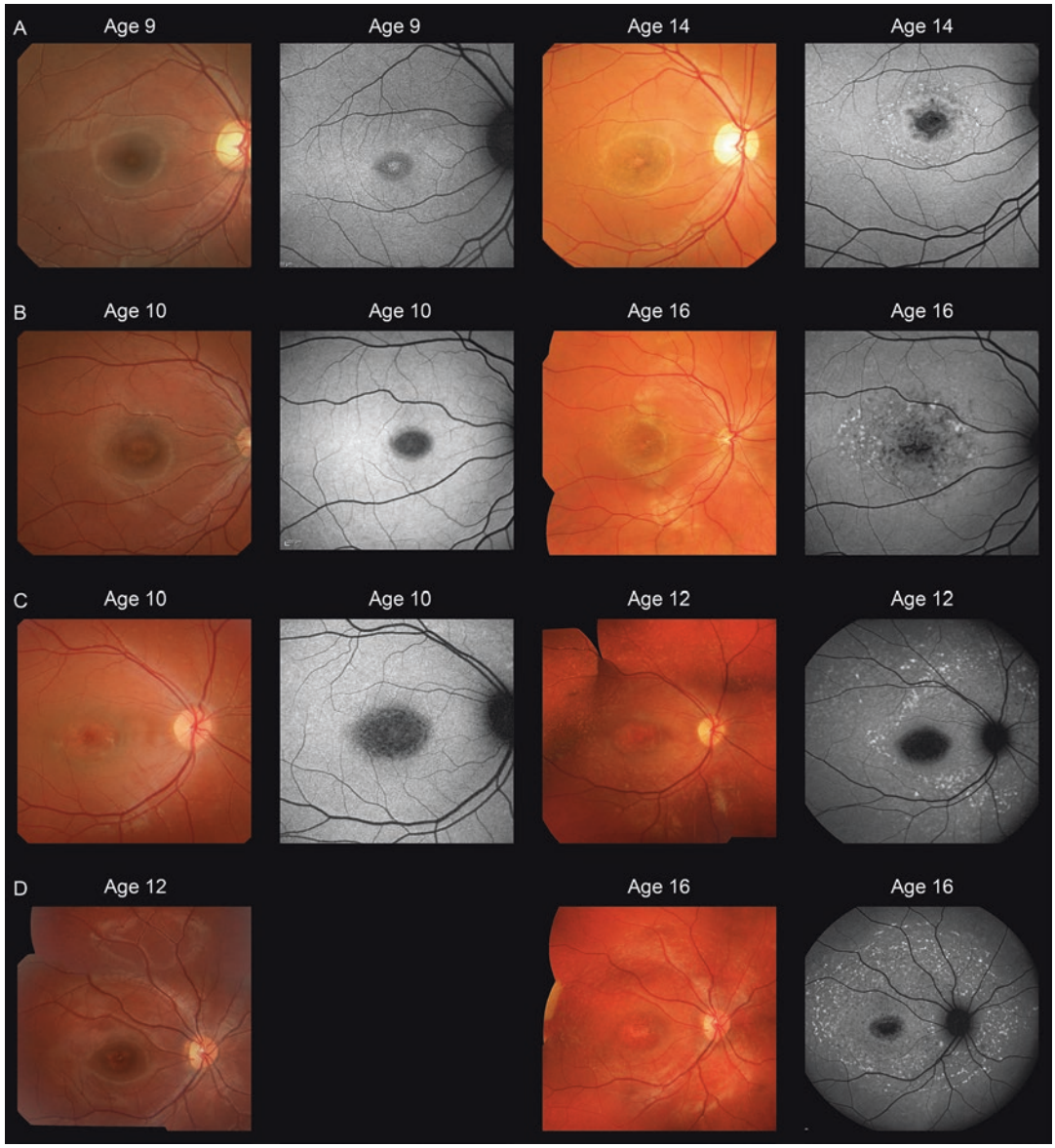

Fig. 23.4 Development of macular atrophy and flecks. At baseline, fundus photography reveals normal or minimal macular abnormalities with evidence of macular abnormality detected by AF. Overtime, marked macular atrophy and macular and/or peripheral flecks are developed, which are recognisable both by fundus photography and AF imaging

phy (65 patients) [51]. The prevalent variants were p.Tyr808Ter (15 alleles), p.Phe2188Ser (12 alleles), p.Ser34-Leu35del (10 alleles), and p. Asn965Ser (10 alleles). All these variants are not described as prevalent variants in Caucasian population.

Fukui et al. reported the disease-causing variants in a Japanese cohort with Stargardt disease and retinitis pigmentosa [52]. The prevalent variants were c.1760+2T>G (five alleles), p. Leu1894Leu (four alleles), p.Leu1938Leu (four alleles), c.5838-11G>A (four alleles), and p. Pro1948Pro (four alleles). These variants are dif-

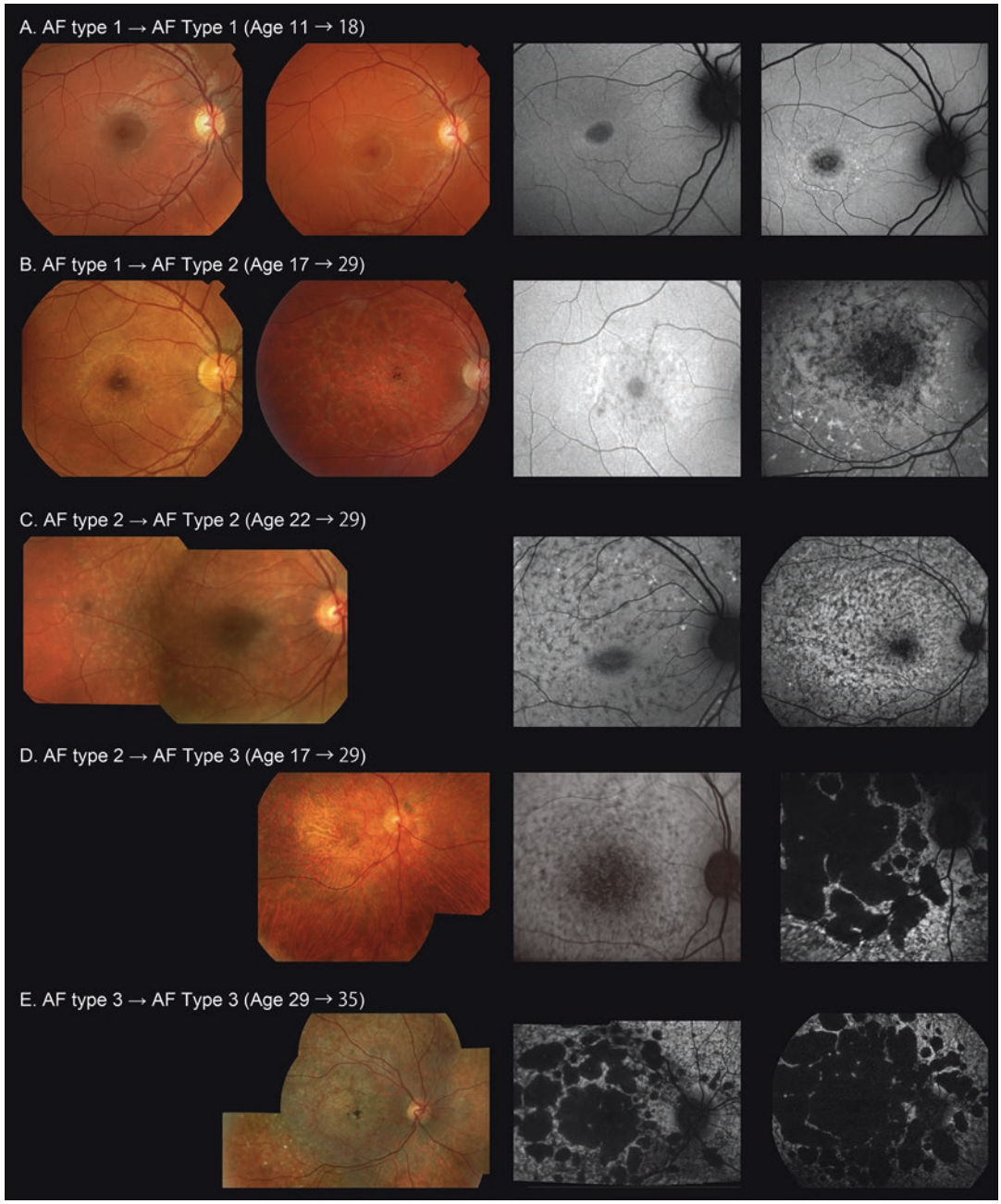

Fig. 23.5 Autofluorescence patterns and progression. AF classification is performed based on the size of the area with low signal and background features (heterogeneous/homogeneous). A severe type shows rapid progression associated with null *ABCA4* variants. Detailed descriptions are presented in Table 23.2. (Fujinami et al. A longitudinal study of Stargardt disease: quantitative assessment of fundus autofluorescence, progression, and genotype correlations. Invest Ophthalmol Vis Sci. 2013)

ferent from those of Chinese population, although clinical presentation of the high proportion was retinitis pigmentosa. Interestingly, these are reports of FS-STGD1 in Japanese population, which could be relatively common in Japanese [10, 53].

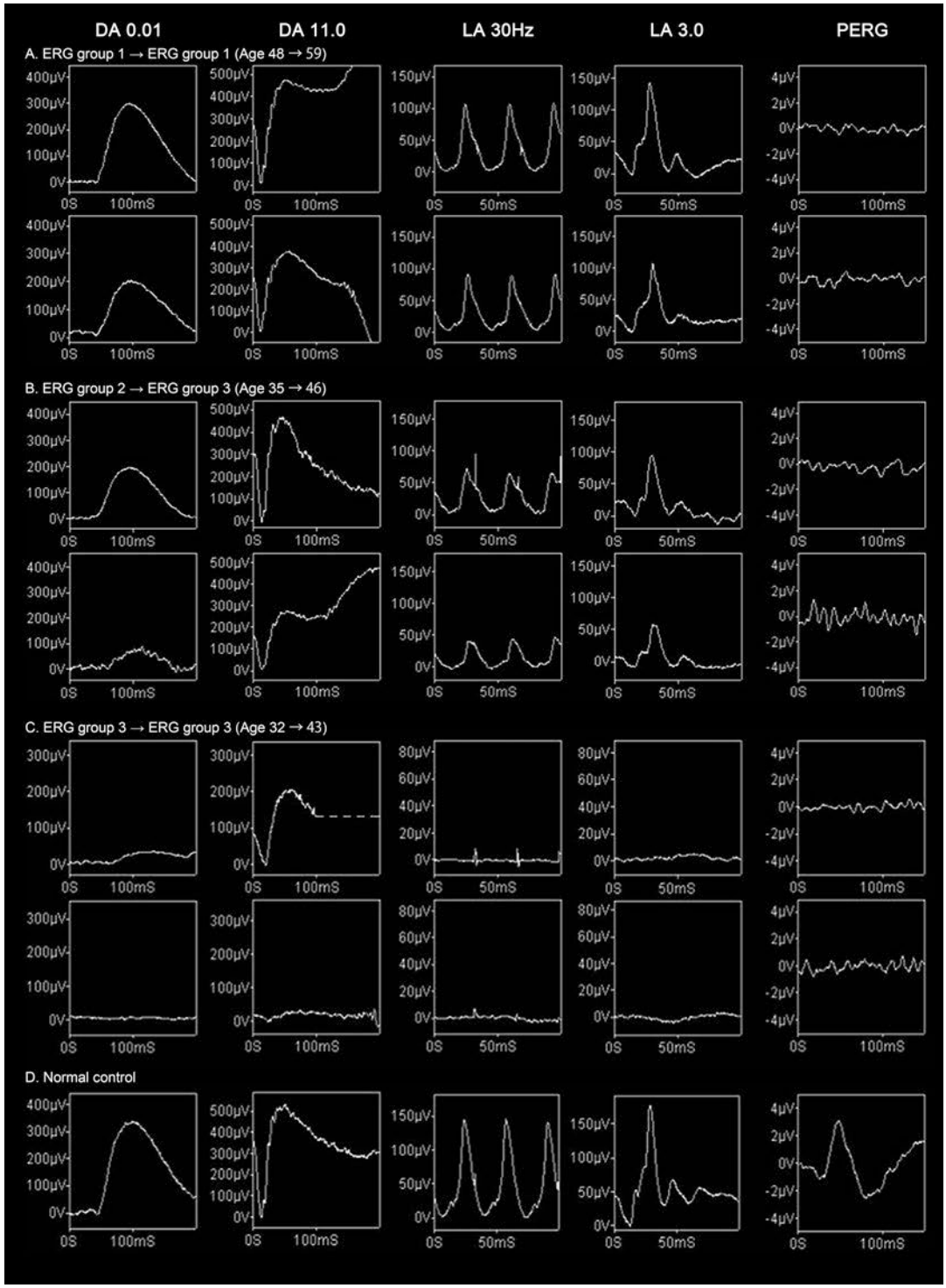

Fig. 23.6 Functional phenotype and electrophysiological progression Full-field electroretinogram (FFERG) and pattern ERG (ERG) of three representative cases from each ERG group and a normal subject are shown. ERG group classification is performed based on the presence of macular dysfunction (abnormal PERG or macular ERG), generalised cone dysfunction (abnormal light-adapted FFERG), and generalised rod dysfunction (abnormal dark-adapted FFERG). Detailed descriptions are presented in Table 23.3. All patients with initial rod ERG involvement demonstrated clinically significant electrophysiological deterioration overtime; only 20% of patients with normal FFERGs showed clinically significant progression. (Lois et al. Phenotypic subtypes of Stargardt macular dystrophy-fundus flavimaculatus. Arch Ophthalmol 2001; Fujinami et al. A longitudinal study of Stargardt disease: clinical and electrophysiological assessment, progression, and genotype correlations. Am J Ophthalmol 2013)

Table 23.1 Classification for fundus appearance in Stargardt disease

Grade 1	Normal fundus
Grade 2	Macular and/or peripheral flecks without central atrophy
Grade 3a	Central atrophy without flecks
Grade 3b	Central atrophy with macular and /or peripheral flecks
Grade 3c	Paracentral atrophy with macular and/or peripheral flecks, without a central atrophy
Grade 4	Multiple extensive atrophic changes of the RPE, extending beyond the vascular arcades

Fujinami et al. Clinical and Molecular Characteristics of Childhood-Onset Stargardt Disease. Ophthalmology 2015

Table 23.2 Classification for autofluorescence pattern in Stargardt disease

Pattern 1	Localised low AF signal at the fovea surrounded by a homogeneous background with/without perifoveal foci of high or low signal
Pattern 2	Localised low AF signal at the macula surrounded by a heterogeneous background and widespread foci of high or low AF signal extending anterior to the vascular arcades
Pattern 3	Multiple areas of low AF signal at posterior pole with a heterogeneous background and/or foci of high or low signal

Fujinami et al. A longitudinal study of Stargardt disease: quantitative assessment of fundus autofluorescence, progression, and genotype correlations. IOVS 2013

Table 23.3 Classification for electrophysiologic findings in Stargardt disease

Group 1	Macular dysfunction (normal full-field ERG)
Group 2	Macular dysfunction with generalised cone dysfunction
Group 3	Macular dysfunction with generalised cone and rod dysfunction

ERG electroretinogram
Lois et al. Phenotypic subtypes of Stargardt macular dystrophy-fundus flavimaculatus. Arch Ophthalmol 2001; Fujinami *et al.* A longitudinal study of Stargardt disease: clinical and electrophysiologic assessment, progression, and genotype correlations. Am J Ophthalmol 2013

Table 23.4 Classification for genotype classification in *ABCA4* retinopathy

Genotype A	Two or more likely deleterious variants
Genotype B	One deleterious variant and one or more missense or in frame insertion/deletion variant(s)
Genotype C	Two or more missense or in frame insertion/deletion variants

Fujinami et al. Clinical and Molecular Characteristics of Childhood-Onset Stargardt Disease. Ophthalmology 2015

Song et al. reported safety and potential efficacy of subretinal transplantation of human embryonic stem cell (hESC)-derived RPE cells in four Asian patients: two with dry age-related macular degeneration and two with Stargardt macular dystrophy [54]. This is the first report of therapeutic trials for STGD1 focusing on Asian population. Otherwise, there are no reports describing disease-causing *ABCA4* variants in a large cohort of Korean population, which appeared on PubMed (https://www.ncbi.nlm.nih.gov/pubmed).

Battu et al. reported three recurrent disease-causing variants in five unrelated patients with STGD1: p.Gly1961Glu (two alleles), p.Thr971Asn (two alleles), and p.Arg2149Ter (two alleles) [55]. Lee et al. described the genotypic spectrum of ABCA4-associated disease in 38 patients of South Asian descent and reported two major prevalent variants: p.Gly1961Glu (17 alleles) and c.859-9T>C (7 alleles) [33]. Interestingly, p.Gly1961Glu is the most prevalent variant in Caucasian population and also frequently found in individuals of Somalian ancestry [56]; thereby the genetic route of this variant could be shared between Asian and Caucasian [11, 16, 17, 57].

Overall, large cohort studies in Asian population are still lacking, and international collaborative studies by Asian Eye Genetics Consortium (AEGC; http://asianeyegenetics.org/) and East Asia Inherited Retinal Disease Consortium (EAIRDc; https://www.fujinamik.com/east-asia-inherited-retinal-disease) are needed, aiming to

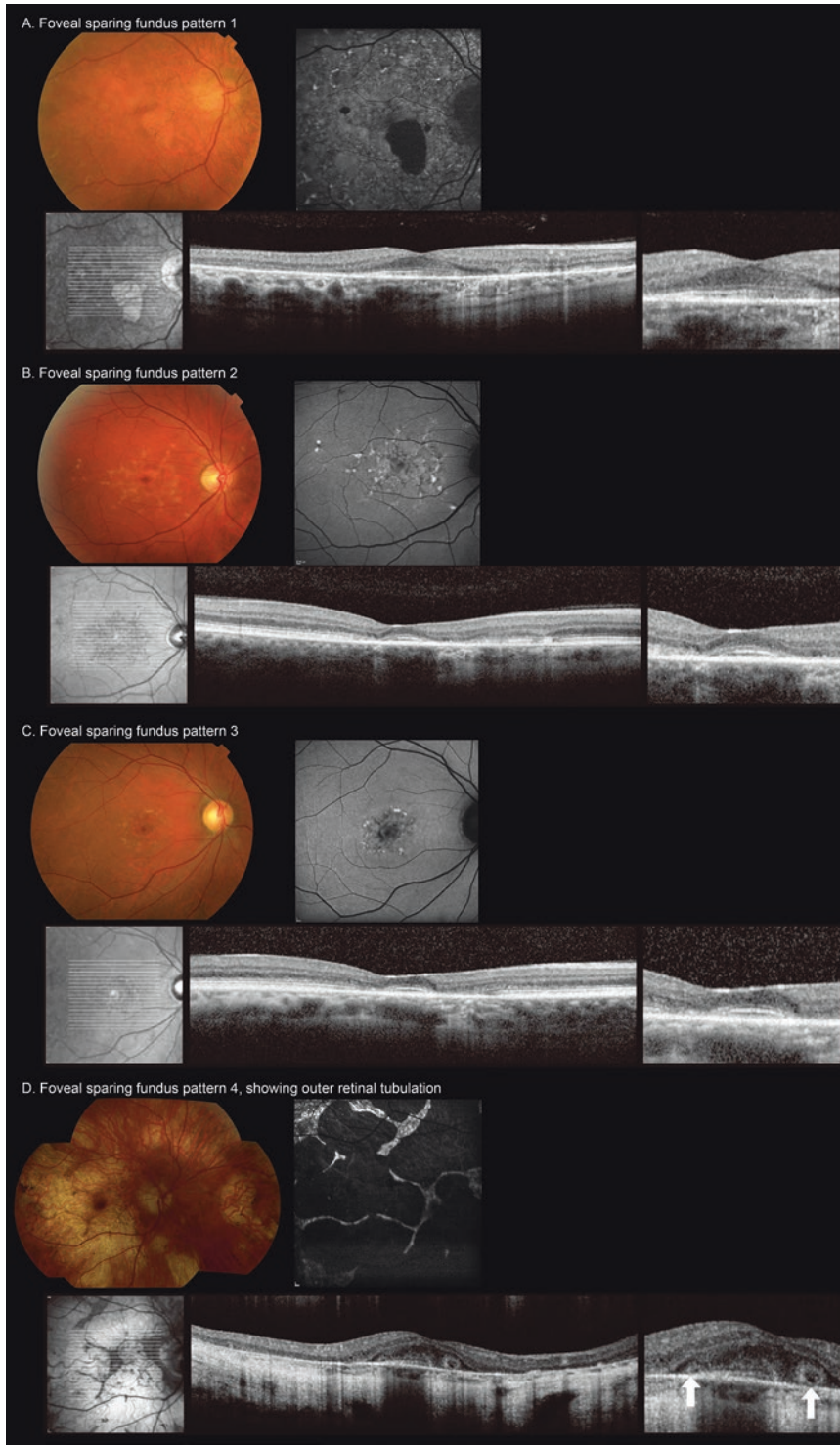

Fig. 23.7 Fundus imaging and spectral-domain optical coherent tomography of foveal sparing phenotype Patients with foveal-sparing STGD1 (FS-STGD1) have preserved visual acuity and relatively maintained foveal structure and function. The outer retinal tabulation (arrowed) at the edge of atrophy is often observed, which suggests the primal damage of this phenotype is RPE/choroid. Detailed descriptions for the pattern classification are presented in Table 23.5 (Fujinami K et al. Clinical and molecular analysis of Stargardt disease with preserved foveal structure and function. Am J Ophthalmol. 2013)

Table 23.5 Fundus pattern for Stargardt disease with foveal-sparing phenotype

Foveal-sparing pattern 1	Patchy parafoveal atrophy surrounded by numerous yellow-white flecks
Foveal-sparing pattern 2	Numerous yellow-white flecks at the posterior pole without atrophy
Foveal-sparing pattern 3	Mottled RPE changes and/or localised parafoveal yellow-white flecks
Foveal-sparing pattern 4	Multiple patchy atrophic lesions, extending beyond the arcades

Fujinami et al. Clinical and Molecular Analysis of Stargardt Disease with Preserved Foveal Structure and Function. Am J Ophthalmol 2013より

develop/apply therapeutic approaches in Asian population.

23.6 Therapeutic Approaches

Several treatment approaches have been developed for STGD1, and clinical trials for gene replacement, stem cell therapy, pharmacological agents, and retinal prosthesis have been ongoing [2, 27, 28].

Gene replacement therapy has been increasingly applied to photoreceptor diseases, aiming to slow or prevent further retinal degeneration. Adeno-associated virus (AAV) vectors have been the major choice for gene transfer system of human gene therapy; however there is a limitation for the size, that is, the ABCA4 gene is larger than the current AAV vector capacity [58]. Considering the larger cargo capacity of lentiviruses, subretinal injection of a lentivirus vector delivering ABCA4 has been developed and is in an ongoing Phase I/II clinical trial (ClinicalTrials.gov Identifier: NCT01367444). There have been no safety concerns in the first three cohorts of subjects with relatively advanced disease and the final cohort with less severe disease now being recruited [28, 59, 60].

A Phase I/II stem cell therapy trial with subretinal transplantation of hESC-derived RPE cells has been ongoing in patients with severe advanced STGD1 (ClinicalTrials.gov Identifier: NCT01469832), given RPE cell dysfunction/loss is believed to precede photoreceptor cell dysfunction/loss in STGD1 [29]. There have been no safety concerns to date.

Several pharmacological agents have been specifically developed that target different aspects of the retinoid cycle and are potentially beneficial in slowing or preventing progression in STGD1 [61]. The aims of these agents are either [1] reducing the formation of toxic products of the retinoid cycle, by reducing delivery of vitamin A or inhibition of various enzymes participating in the cycle, or [2] directly targeting toxic metabolites such as A2E. Visual cycle modulators are candidates for the former treatment [62–64]. A Phase II clinical trial with a chemically modified vitamin A, which does not dimerise and stops N-ret-PE and A2E formation, has been ongoing (ClinicalTrials.gov Identifier NCT02402660) [65–67].

23.7 Conclusion

STGD1 is one of the most common causes of inherited retinal disease associated with *ABCA4* disease-causing variants. STGD1 is highly heterogeneous both phenotypically and genetically, and investigations have been performed to understand the underlying disease mechanisms, which allows to conduct multiple clinical trials. However, these trials have been performed mostly in Caucasian population, and further robust longitudinal studies, probing genotype–phenotype and structure–function associations in Asian population, are crucial in order to provide improved prognostication and genetic counselling, as well as to initiate therapeutic trials.

Table 23.6 Prevalent *ABCA4* variants in Asian population

Reports	Ethnicity	Number of affected subjects	Recruitment criteria	Prevalent variants			
				Variant 1	Variant 2	Variant 3	Variant 4
Jiang et el. Screening of ABCA4 Gene in a Chinese Cohort With Stargardt Disease or Cone-Rod Dystrophy With a Report on 85 Novel Mutations. Investigative Ophthalmology & Visual Science January 2016	Chinese	161	161 unrelated patients with STGD1 (96 patients) and CRD (65 patients)	c.2424C>G, p.Tyr808Ter (15 alleles)	c.6563 T>G, p. Phe2188Ser (12 alleles)	c.101_106delCTTTAT, p.Ser34-Leu35del (10 alleles)	c.2894A>G, p.Asn965Ser (10 alleles)
Fukui T, et al. "ABCA4 gene mutations in Japanese patients with Stargardt disease and retinitis pigmentosa. " Investigative Ophthalmology & Visual Science 2002.	Japanese	110	110 unrelated patients with STGD1 (10 patients), arRP(96 patients), and CRD (4 patients)	c.1760+2T>G, splice site alteration (5 alleles)	c.5682G>C, p.Leu1894Leu (4 alleles) c.5814A>G, p.Leu1938Leu (4 alleles) c.5838-11G>A, splice site alteration (4 alleles) c.5844A>G, p.Pro1948Pro (4 alleles)	c.6285T>C, p.Asp2095Asp (3 alleles)	c. 5644A>G, p. Met1882Val (2 alleles)
Song, et al. Treatment of Macular Degeneration Using Embryonic Stem Cell-Derived Retinal Pigment Epithelium: Preliminary Results in Asian Patients. Stem Cell Reports 2015.	Korean	2	2 unrelated patients with STGD1	c.983A>T, p.Glu328Val (1 allele) c.1933G>A, p.Asp645Asn (1 allele) c.3106G>A, p.Glu1036Lys (1 allele) c.2894A>G, p.Asn965Ser (1 allele) c.4972A>C, p.Ser1658Arg (1 allele)			

(continued)

Table 23.6 (continued)

Reports	Ethnicity	Number of affected subjects	Recruitment criteria	Prevalent variants			
				Variant 1	Variant 2	Variant 3	Variant 4
Battu, R, et al. Identification of Novel Mutations in ABCA4 Gene: Clinical and Genetic Analysis of Indian Patients with Stargardt Disease. Biomed Research International 2015.	Indian	5	5 unrelated patients withSTGD1	c.5882G>A, p.Gly1961Glu (2 alleles)	c.2912C>A, p.Thr971Asn (2 alleles)	c.6445C>T, p.Arg2149Ter (2 alleles)	c.4918C>T, p.Arg1640T (1 allele)rpc.514G>A, p.Gly172Ser (1 allele) c.570+1G>A, Splice site alteration (1 allele) c.2616C>G, p.Tyr872Ter (1 allele)
Lee W et al. Genotypic spectrum and phenotype correlations of ABCA4-associated disease in patients of south Asian descent. Eur J Hum Genet. 2017.	South Asian	38	Not described	c.5882G>A, p.Gly1961Glu (17 alleles)	c.859-9T>C, splice site alteration (7alleles)	c.93G>A, p.Trp31Ter (2 alleles), c.91T>C, p. Trp31Arg (2 alleles), c.1531C>T, p.Arg511Cys (2 alleles)c.2894A>, p.Asn965Ser (2 alleles), c.2966T>C, p.Val 989Ala (2 alleles), c.5917del, p. Val1973Ter (2 alleles)c.6191C>T, p.Ala2064Val (2 alleles), c.6729+5_6729+19del, splice site alteration (2allele)c.179C>T, p.Ala60Val/c.3830C>T,p. Thr1277Met (complex; 2 alleles)	

CRD cone–rod dystrophy, *STGD1* Stargardt disease, *arRP* autosomal recessive retinitis pigmentosa

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Retinoblastoma Genes in Chinese Studies

24

Bi Ning Zhang, Yuning Jiang, Wai Kit Chu, Winnie W. Y. Lau, Simon T. C. Ko, Kwong Wai Choy, Calvin C. P. Pang, Guy L. J. Chen, and Jason C. S. Yam

Abstract

Retinoblastoma is the commonest pediatric intraocular malignancy across most ethnic populations. Its genetic basis follows a two-hit model, in which two events are needed to inactivate both alleles of the disease-causative gene, *RB1*. Loss of heterozygosity (LOH) is a major driving force to inactivate the whole *RB1* gene. Epigenetic modifications such as DNA methylation involving different genes and microRNA (miRNA) expressions also play important roles in retinoblastoma tumorigenesis. Functionally, as *RB1* is important in maintaining chromosomal stability and cell cycle progression, loss of its function would underlie aberrations in other chromosomal regions. In addition, genes other than *RB1* have been identified as direct or indirect causes of retinoblastoma tumorigenesis. In this chapter, we review the major genetic studies of retinoblastoma that have been conducted in Chinese. Contrasts with other ethnic groups will be made. These studies help us to understand the mechanisms of inactivating *RB1*, its functional consequences, and maintaining genome stability. Evidences have also been obtained on epigenetic contribution to retinoblastoma, especially by DNA repair genes. On the basis of the genetic and epigenetic findings, development of potential alternative therapy for retinoblastoma will be discussed, together with key issues in attempts of awareness campaign for early detection and genetic counselling.

Keywords

Retinoblastoma · *RB1* · LOH · Methylation · Chromosomal instability

B. N. Zhang · Y. Jiang · W. K. Chu · C. C. P. Pang
J. C. S. Yam (✉)
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Kowloon/Hong Kong, China
e-mail: yamcheuksing@cuhk.edu.hk

W. W. Y. Lau
Hong Kong Eye Hospital, Hong Kong, China

S. T. C. Ko
Department of Ophthalmology, Tung Wah East Hospital, Hong Kong, China

K. W. Choy
Department of Obstetrics and Gynaecology, The Chinese University of Hong Kong, Hong Kong, China

G. L. J. Chen
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Kowloon/Hong Kong, China

Department of Ophthalmology and Visual Sciences, Prince of Wales Hospital, Hong Kong, China

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24.1 Retinoblastoma Epidemiology

Retinoblastoma is the most common childhood intraocular cancer affecting all populations. It accounts for 3% of all cancers occurring in children younger than 15 years old [1]. Its incidence is approximately 40–60 per million live births worldwide, which corresponds to 1 per 15,000–25,000 live births. In the United States and Europe, the age-adjusted incidence is 2–5 cases per 1 million children (approximately 1 in 14,000–18,000 live births) [2]. The disease has no validated geographic or populational differences in incidence. The greatest burden is recorded in large populations that have high birth rates, such as Africa, with reportedly approximately six to ten cases per one million children [2]. Population-based studies have been conducted in Asia. Singapore has reported an incidence of 1 in 15,789 live births [3]. In Taiwan it was 1 in 21,691 live births during 1979–2003 [4] and 1 in 17,373 live births during 1998–2011 [5]. Incidence of 1 in 18,000 live births had been reported in Hong Kong [6]. According to hospital records from 2009 to 2014, there were 35 eyes diagnosed as retinoblastoma in Hong Kong, approximately 3–9 new cases per year (unpublished data). There is no strong geographic difference in incidences of retinoblastoma by gender or laterality. However, studies from Brazil and Mexico have reported an inverse association between the incidence of retinoblastoma and socioeconomic level index [7, 8]. In the United States, an increased incidence has been correlated with low levels of maternal education and poverty [9]. Table 24.1 summarizes the age-standardized incidence rates (ASIR) of retinoblastoma in Asia. Some countries including China and Japan employed survival rate or the total incidence number for statistical analysis of retinoblastoma, and these data are therefore not included.

Table 24.1 Age-standardized incidence rates (ASIR) of retinoblastoma in Asia

Region	Race/ethnicity	Case no	ASIR/million	Study period	References
United States[a]	White	346	2.8	2000–2010	[104]
	Hispanic	274	3.6		
	Black	119	3.5		
	API[b]	75	3.1		
	AI/AN[b]	10	2.1		
	Total	824	–		
Singapore	Asian	46	2.4 (<9 years)	1968–1995	[3]
			11.1 (<5 years)		
India	Asian	82	7.6 (boys)	1990–2001	[14]
			7.9 (girls)		
Korea	Asian	411	4.6	1999–2011	[105]
Taiwan	Asian	380	8.58 (0–4)	1979–2003	[4]
			0.28 (5–9)		
			0.05 (10–14)		
Thailand	Asian (Khon Kaen)	31	4.4	1990–2009	[106]
	Asian (Chiang Mai)	20	4.0		
	Asian (Songkhla)	24	4.6		
Jordan	Asian	40	9.32	2006–2010	[107]
Saudi Arabia	Asian	597	7.7 (<5 years)	1983–2012	[108]
			3.5 (<15 years)		
Vietnam	Asian	13	9.1 (boys)	1995–1997	[109]
			4.1 (girls)		

[a]The Asian population in this area is counted
[b]API, Native Hawaiian/Asian-Pacific Islander; AI/AN, American Indian/Alaska native

24.2 Clinical Presentation and Complications

Retinoblastoma is a cancer of very young children. In our Hong Kong Chinese cohort, approximately 60% were diagnosed before 2 years and 95% before 5 years [unpublished data]. About 68% of cases present with leukocoria, which is the white reflex in the pupil. This manifestation is often firstly noticed by parents under dim illumination or flash photography. It is the most common presentation of retinoblastoma in Hong Kong [6]. The second most frequent sign is strabismus, which accounts for 10–20% of cases and usually correlates with macular involvement. Some children may also present with pain, glaucoma, poor vision, orbital cellulitis, anterior uveitis, and buphthalmos. As the tumor progresses, some patients may develop orbital or metastatic systemic extra-central nervous system (CNS) disease. Patients with bilateral disease usually present at a younger age, in the first 12 months after birth. The median age of presentation is 18 months in our Hong Kong cohort.

Retinoblastoma growth is underneath the retina layer and toward the vitreous. With its progression, the choroidal or scleral layer and optic nerve may be entangled. Progression through the ocular coats results in invasion of the tumor into orbit, choroid, anterior chamber, and even to systemic circulation. Tumor progression through the optic nerve leads to systemic and central nervous system metastasis. Saving patients' life is the priority of managing retinoblastoma. Compared with the developed world where most cases are detected early, retinoblastoma is often detected late resulting in an enlarged eye with locally invasive disease in developing countries. In Hong Kong, the survival rate is 95.1% during 1980–2009, with 5 deaths among 104 patients, whereas the survival rate in the United States approaches 100%. However the survival rate is lower in many other parts of the world: 80–89% in South America [10], 83% in Iran [11], 81% in China [12, 13], 48% in India [14], and 20–46% in Africa [15, 16]. Consequently 3000–4000 annual deaths were estimated globally [17]. Notably, if survival rates of these relatively lower areas approaching those of Europe, the United States, and Japan, deaths of children due to retinoblastoma could be substantially reduced worldwide [18]. The prognosis of retinoblastoma also depends on the stage of presentation at diagnosis. According to our hospital record in Hong Kong from 2009 to 2014, 34% of cases were at late stage at presentation. Early diagnosis is essential to improve prognosis, especially for children with a positive family history. For these children, screening by fundus examination should be performed under anesthesia at regular intervals. For surviving children that grow up and become parents, their infants should have a dilated eye examination in the first month of life. Genetic testing of *RB1* should be conducted. Infants with a positive genetic test should be examined on a monthly basis. For infants who do not develop retinoblastoma but with a positive genetic test, monthly exams should be conducted continually throughout the first year. Siblings of patients should also be examined continually until age 3–5 years, and it is important to check the *RB1* mutation status [18].

24.3 Introduction of the *RB1* Gene

Before the retinoblastoma-causative gene *RB1* was identified, a two-hit hypothesis was proposed to describe the genetic basis of retinoblastoma by Knudson in 1971 based on the observation of 48 retinoblastoma cases [19]. Two mutation events are needed to develop retinoblastoma. The genomic origin of the first mutation event also affects the heredity [19]. For inherited retinoblastoma, a germline mutation is required to predispose risk in the offspring. In combination with a second spontaneous mutation, the complete inactivation of the disease-causing gene would lead to development of retinoblastoma in the offspring. For the non-hereditary form, the two spontaneous mutations can occur in somatic cells of the offspring. In the early 1980s, several studies found close association of retinoblastoma with abnormities on chromosome 13, where the *RB1* locus was identified [20–22]. The clear relationship between *RB1* locus with retinoblastoma and other malignancies indicated the existence of

tumor-suppressor genes [23, 24]. In 1986, a DNA segment containing *RB1* locus at 13q14 was isolated, and deletions in the region were found in retinoblastoma [25]. In 1987, two studies reported the transcripts of *RB1* gene with abnormal mRNA expression in some cases of retinoblastoma [26, 27]. The RB amino acid sequence was subsequently predicted [26] and deletion hotspots identified [27]. These studies provided molecular evidence for Knudson's two-hit hypothesis and suggested *RB1* gene as a causative gene for retinoblastoma. In an experimental model, an exogenous normal *RB1* gene was introduced into RB-inactivated tumor cells by retrovirus to rescue the tumorigenesis phenotype in cell lines and in nude mice [28]. Thus, *RB1* has been validated as a bona fide tumor-suppressor gene.

24.4 *RB1*: The Key Player

The *RB1* gene (NM_000321) spans around 200 kb. The protein-coding transcript (*RB1–201*) contains 27 exons with some large introns [29]. The exon 13–17 region is frequently mutated in multiple tumor types and is denoted as the potential "hotspot" [27, 29]. Human RB protein (pRB) consists of 928 amino acids and is a member of "pocket protein" family [30]. pRB can be divided into three major domains: central pocket domain (aa 379–791), pRB C-terminus domain (RbC; aa 792–928), and pRB N-terminal domain (RbN; aa 1–378). The central pocket domain is essential to its function [31] and is composed of A and B cyclin-like subdomains [31]. This central pocket is the target to be inactivated by viral oncoproteins, such as human papilloma virus E7 [31] and SV40 large T antigen [32]. The pRB C-terminus domain contains five cyclin-dependent kinase (CDKs) phosphorylation sites [33], which are important for the recruitment of and modification by cyclins and CDKs. Such activity is involved in regulation of cell cycle progression. Two cyclin-like folds form a globular entity at the pRB N-terminal domain [34]. This domain interacts with the central pocket domain to regulate the conformation of pRB [34]. Phosphorylation at the sites T373, S608/S612, and T821/T826 could promote pRB intramolecular interactions and conformation change [35]. The protein structure (Fig. 24.1a) underlies the function and the post-translational modification of pRB. Deciphering the protein structural information has greatly facilitated our understanding of the multifunctionality of this tumor-suppressor protein [36].

24.5 *RB1* Inactivation

Germline *RB1* mutations often give rise to bilateral retinoblastoma, while individual retinal cells acquiring somatic changes on both alleles result in unilateral tumor. There are several possibilities causing inactivation of the *RB1* alleles. In most circumstances, single nucleotide mutations or small deletions account for the inactivation of the first *RB1* allele [37]. When the first mutation happens in the constitutional germline cell, all cells in the body are inherited with one mutated *RB1* allele. Nature of the second mutation can be variable, including another mutation, which can be de novo, epigenetic modifications, or loss of heterozygosity (LOH). In diploid organisms, there are several ways to gain LOH. For short genomic fragments less than 2 kb, recombination via gene conversion or double crossovers between chromosomes is the main source of LOH [38]. Long-range LOH is often related to repairing DNA breaks which are deleterious [38]. To repair DNA breaks, the required DNA strand integrates into the homologous chromosome and initiates the complementary DNA sequence to repair the broken region, the phenomenon of break-induced replication. The integrated strand could replicate DNA to the end of the chromosome, causing a long-track LOH between the DNA breakage site and the end of the chromosome. DNA double-strand break (DSB) repair between homologs at the *RB1* locus could lead to more than 100-fold induction of LOH upon DSB induction [39].

Our study in a Hong Kong Chinese cohort of 42 sporadic retinoblastoma patients identified 15 *RB1* mutations in 16 patients, 8 of them germline mutations. There were nine stop codons and four splice site variations, the former leading to truncations of pRB affecting its

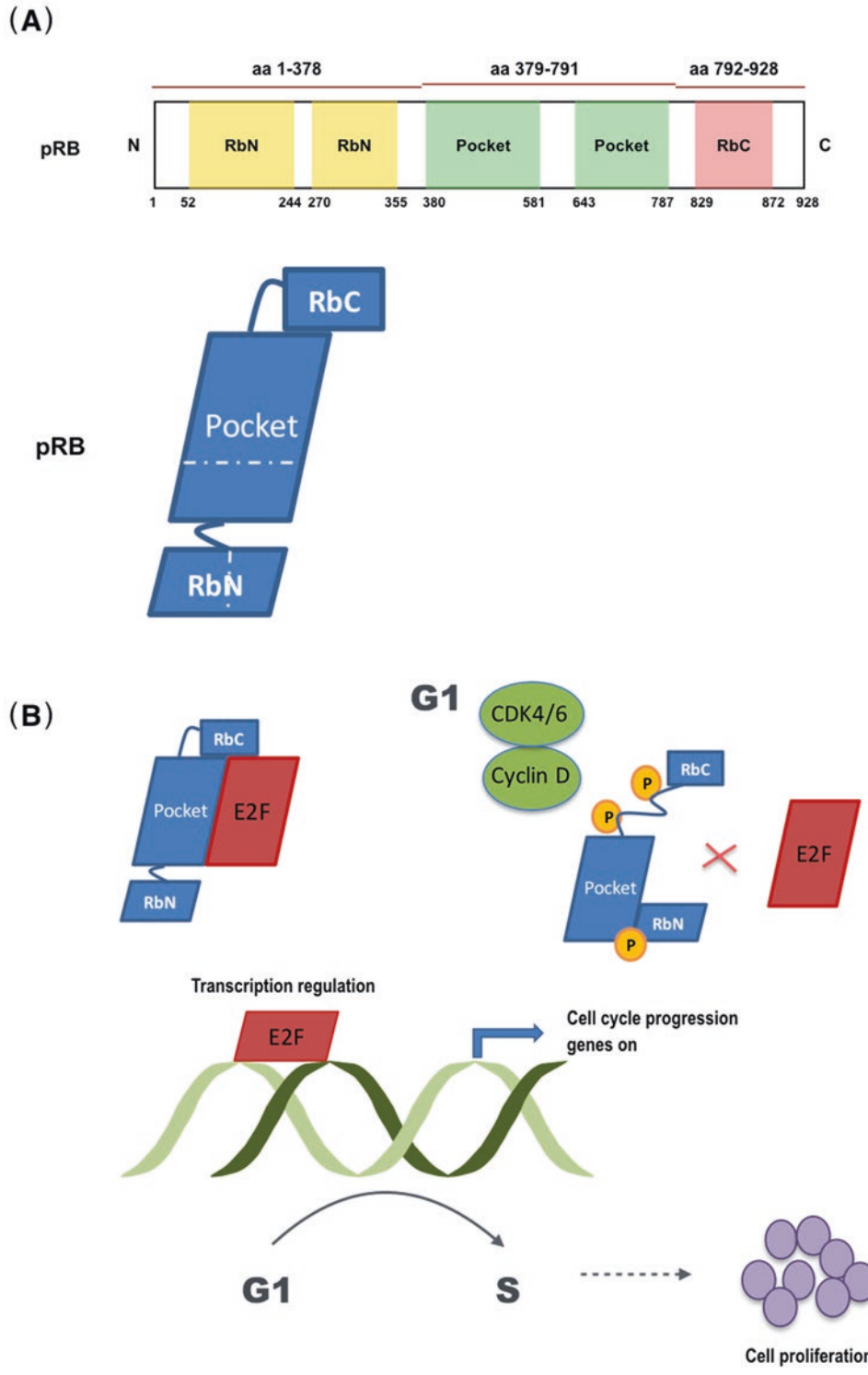

Fig. 24.1 (**a**) Retinoblastoma protein (pRB) structure. **(b)** In G1 phase, highly expressed cyclin D activates CDK4 and CDK6. Activated CDKs phosphorylate pRB, causing pRB conformation change and dissociation of the binding transcription factor E2F. Released E2F induces cell cycle-related gene expression and cell enters S phase

central large pocket domain [40]. No methylation across the CpG dinucleotides of the *RB1* promoter among all 42 patients was found. Among 15 patients who had both normal and retinoblastoma tissues available for analysis of 3 microsatellite markers (D13S153, D13S156, and D13S128), 9 (60%) of them showed LOH at 13q14, and they did not express nuclear RB. The results thus showed inactivation of the *RB1* gene is mainly caused by loss-of-function mutations and LOH, but not epigenetic effect of promoter methylation [40]. In a Chinese retinoblastoma cohort in Beijing, in the north of China, 10 of the 47 patients had *RB1* exon mutations, while 4 had CpG island hypermethylation in the *RB1* promoter. LOH was also detected in 60% of 30 cases, at loci D13S153, D13S262, and D13S284 [41]. In another cohort in Chongqing in Western China, screening of LOH with 14 microsatellite markers in 16 sporadic retinoblastoma patients showed 12 (75%) had LOH at 13q14 [42]. Similar studies had been conducted in other ethnic populations. In Russia, a retinoblastoma molecular screening study involving 45 families identified 23 *RB1* mutations, and 9 (20%) exhibited abnormal methylation status in the *RB1* promoter and 70% showed LOH [43]. In an Indian cohort, 72.9% of 54 retinoblastoma patients showed LOH of *RB1* [44]. These studies show that LOH plays an important role in tumorigenesis of retinoblastoma.

A rare cause of *RB1* inactivation is chromothripsis, which is a phenomenon of tens to hundreds of rearrangements of genome sequence happening at one time [45]. Focal chromothripsis was found on chromosome 13 in three retinoblastoma cases [46]. More studies are needed to investigate the impacts of chromothripsis in retinoblastoma.

24.6 Mutations in *RB1*

A database (RBGMdb) was constructed form meta-analysis of 932 *RB1* mutations, showing 40% of mutations located in 16 putative mutation hot spots from exon 12 to exon 23, while others scattered throughout the gene [47]. Splicing mutations and late-onset of the disease were mostly found in bilateral retinoblastoma patients, while children with nonsense, frameshift, or missense mutations had relatively early onset. While ethnic segregation of mutations was observed [47], similar relations were found in a study on 85 Chinese patients, with null mutations giving a higher percentage of bilateral (96.8%) and early diagnosed (10.7 months) retinoblastoma than in-frame mutations (66.7% and 13.5 months, respectively) [48]. Among 1173 Dutch retinoblastoma patients, *RB1* mutations occurred in 92% bilateral or familial patients, and about 10% unilateral sporadic patients [49]. About 80% of *RB1* mutations are nonsense or frameshift mutations causing truncated pRB [50, 51]. A small proportion of retinoblastoma patients, 2–5%, do not carry a *RB1* mutation, and some possess amplified copy numbers of other oncogene [49, 52]. There are also *RB1* mutation carriers that do not develop retinoblastoma, but offspring from such unaffected parents may inherit a potential disease-causing mutation. There are a small proportion of low-penetrance families. For clinical assessments and genetic counselling, detailed information of the *RB1* sequence and inheritance patterns have to be available [51].

24.7 The Classical Cell Cycle Regulatory Pathway

The lack of functional pRB can predispose patients to retinoblastoma development. pRB regulates the cell cycle progression through controlling the transcription process [53] (Fig. 24.1b). In the four phases of a cell cycle, G1, S, G2, and M phase, the first three phases are interphase, during which the cell is prepared for cell division mainly by doubling the genomic materials and undergoing physiological metabolisms. The M phase is the mitosis phase, during which chromosomes and cytoplasm divide into two daughter cells. Other proteins are also involved to regulate progression of the cell cycle, including cyclins and the cyclin-dependent kinases (CDKs). Cyclins are expressed at specific stages of the cell cycle [54]. For example, in G1 phase, cyclin D is expressed

in a higher level and activates CDK4 and CDK6, which are able to phosphorylate pRB. Highly phosphorylated pRB was found in rapid proliferating cells, while dephosphorylation of pRB induced cell growth arrest [55, 56]. The phosphorylated pRB formed a complex with the transcription factor E2F [57, 58], while the uncomplexed form of E2F1 could drive the quiescent cells to enter S phase [59]. Based on these observations, a model has been proposed in that when there is a loss of pRB activity, E2F is able to activate the transcription of other genes necessary for DNA synthesis and cell cycle progression [60].

24.8 *RB1* and Chromosomal Instability

Inactivated *RB1* initiates the onset of retinoblastoma by dysregulating cell cycle progression. Cancer has several hallmark features: replicative immortality, invasion and metastasis, angiogenesis, cell death avoidance, and sustainable proliferation [61, 62]. Therefore, there is a need to understand the relationships of pRB inactivation and these phenomena. Other than regulation of the cell cycle, pRB is also involved in maintaining genomic stability. For sporadic cancers, one cause of genomic instability is DNA replication stress [63]. As mentioned above, in *RB1*-inactivated cells, the RB-free E2F can promote the expression of cell cycle progression genes and lead to premature entering into S phase. In these cells, the existing nucleotide pool may not be sufficient in availability for the required high-level DNA replication [64], which could result in accumulating DNA replication stress. Nucleotide deficiency during S phase would lead to inadequate replication of the genome, leaving some unreplicated DNA regions as single-strand breaks (SSBs). These SSBs could be further converted into double-strand breaks (DSBs) by a nearby DNA replication fork [65].

There are essentially two cellular strategies to repair these DSBs: homologous recombination (HR) and nonhomologous end joining (NHEJ). In the HR pathway, the ends of DSBs integrated into the homologous sequence to repair the DSBs [66]. In the NHEJ pathway, the ends of DSBs are ligated directly to each other [67]. As NHEJ pathway does not require the terminal sequence homology, inaccurate repair and even DNA sequence loss could happen at the breakage sites. Thus, NHEJ is considered to be "error-prone." On the contrary, HR is considered to be "error-free" as the homologous sequences are highly similar to the original broken DNA. Since mammalian cells prefer to use NHEJ for DSB repair [68], the chance of chromosomal instability raised tremendously in *RB1*-deficient cells. Therefore, *RB1* mutations can be considered as the primary driving force of tumorigenesis. The *RB1*-mutated cells could then accumulate additional mutations that could lead to other features of cancer development.

24.9 Recurrent Chromosomal Aberrations in Retinoblastoma

In the human genome, there are regions known as fragile sites that are difficult to be replicated during S phase. These fragile sites are prone to form gaps and breakages, which could be observed during mitosis, particularly when under replication stresses [69]. In one study constitutional chromosomal instability investigation at fragile sites was conducted in 36 retinoblastoma patients, 2 of them hereditary form and the rest sporadic. High prevalence of chromosome breakages at 3p14, 6p23, 13q14, 13q22, and 16q22-23 loci was identified. Tumor karyotype analysis found that 50% of this cohort showed structural aberrations on chromosomes 13, 1, 6, and 16 [70]. Our study on 15 microdissected retinoblastoma samples showed 73% having chromosomal changes at one or more loci on chromosomes 19, 20, 21, 22, and X [71]. Single allele loss was more common on chromosome 19 (33%) and 20 (27%). The most frequent recurrent allelic loss happened on 19q13 between D19S902 and D19S571, suggesting this locus may be associated with tumorigenesis [71]. Comparative genomic hybridization (CGH) studies on retinoblastoma karyotype have been performed and recurrent changes documented [72, 73].

24.9.1 Chromosome 1q

In 179 retinoblastoma samples, 95 (53%) were found to have gained an additional copy of chromosome arm 1q (+1q) [72]. Although occurrence of +1q is common in other cancers, its presentation in retinoblastoma is different. Specifically, the +1q was not highly expressed, and the additional regions were often distributed along the whole chromosome arm. *LRRN5* (1q32.1) was found overexpressed in half of tumor samples by Southern blotting [74]. The protein LRRN5 belongs to the leucine-rich repeat superfamily and possesses cell adhesion or signal transduction functions. *KIF14* (1q32.1) got a 100- to 1000-fold overexpression in 90% retinoblastoma retinas than normal fetal retinas [75]. KIF14 is a kinesin family member and works as a microtubule motor protein. Other genes in this region were expressed differentially in various studies including *CENPF, ENSA, HTCD37, SYT11, DAR, SNX27, JTB, LASS2, SYT11, MDM4*, and *ZNF281* [76].

24.9.2 Chromosome 2p

The transcription factor *MYCN* (2p24.1), in chromosome 2p, has been reported as a strong prognostic indicator of neuroblastoma, and its expression has been found elevated in various cancers [77]. About 10–200 copies of *MYCN* amplification have been found in retinoblastoma samples and the Y79 retinoblastoma cell line. *MYCN* expression was highly elevated in all ten retinoblastoma samples examined in a study [78].

24.9.3 Chromosome 6p

Isochromosome 6p [i(6p)], with chromosome arm 6q lost and replaced by duplicating the 6p arm, is unique in retinoblastoma. Unbalanced structural abnormality and i(6p) were found in 60% retinoblastomas [79]. Despite i(6p), 6p22 gain was seen in more than 50% retinoblastoma by CGH studies [80]. By using quantitative multiplex PCR, the most commonly gained bona fide oncogenes in this area were *DEK*, a DNA-binding protein, and *E2F3*, a pRB-regulated target transcription factor [81].

24.9.4 Chromosome 13q

Gain and loss of chromosome 13q have been found in CGH studies. The minimal common region of gain (MRG) was enriched in 13q32-34 region [82]. However, no candidate gene other than *RB1* has been identified in this region.

24.9.5 Chromosome 16q

By the combination of LOHand QM-PCR, and comparison between tumors and normal retinas, the *CDH11* gene was found mutated in more than 40% of retinoblastoma samples in two separate studies [83, 84]. This gene encodes an integral membrane protein, which functions in cell-cell adhesion. It may be involved in the infiltration of retinoblastoma cells into the optic nerve [85]. Another gene *RBL2* (16q12.2) has been found lost in some retinoblastoma patients [86]. When the retinoblastoma cell line WERI was treated with 5-Aza-dC, a DNA methylation inhibitor, the expression of *RBL2* was restored [86]. This result indicated that RBL2 expression was epigenetically suppressed in retinoblastoma.

24.10 Epigenetics in Retinoblastoma

The *RB1* gene is subjected to numerous epigenetic modifications. Stable genome has been observed in some patients, while epigenetic dysregulations have also been reported. Epigenetic modification clusters are summarized in Fig. 24.2.

24.10.1 Methylation

An early study on methylation status of *RB1* promoter in a cohort of 21 patients found association

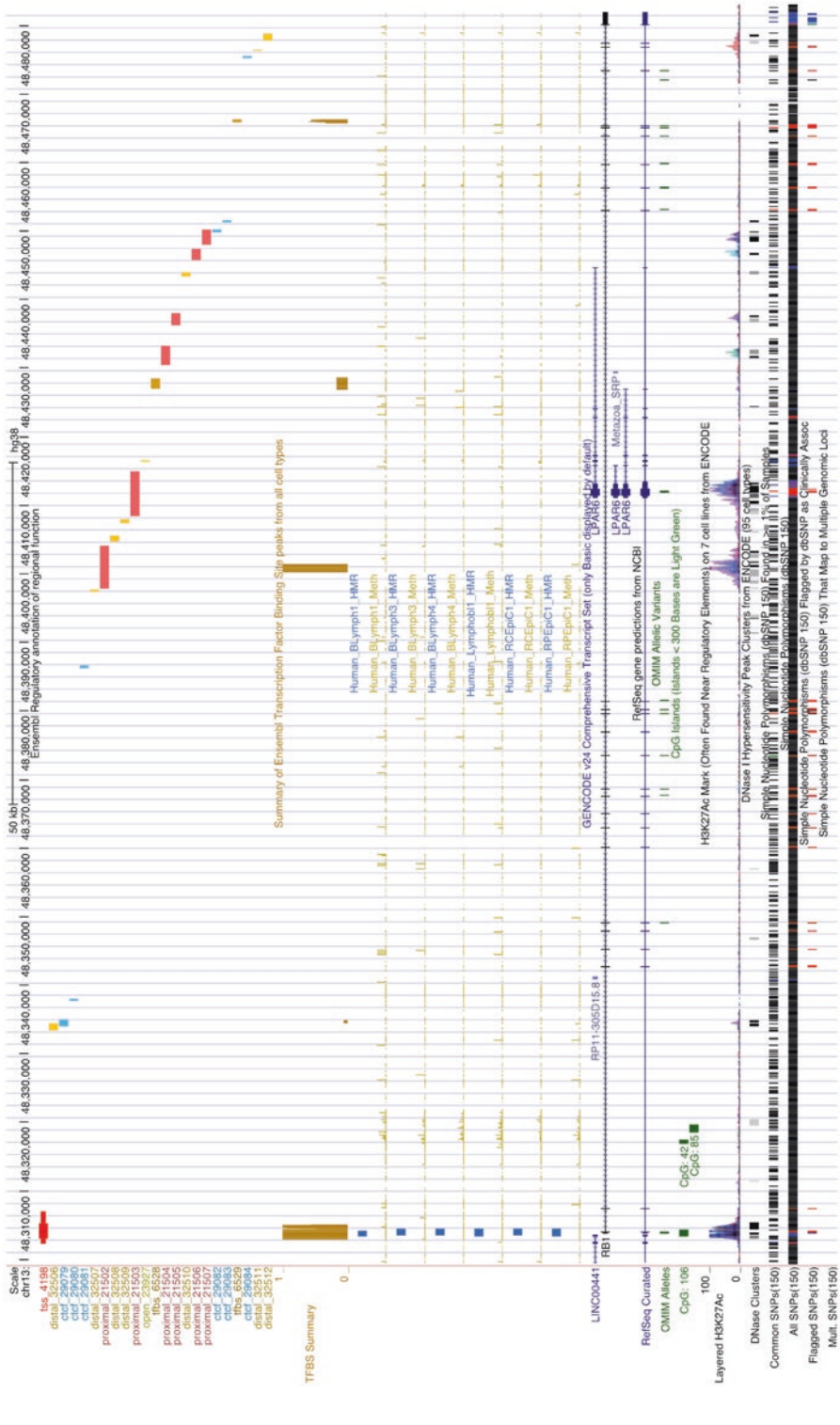

Fig. 24.2 Summary of *RB1* information on Ensembl transcription factor binding sites [110–112], ENCODE methylation data [113], CpG island tracks [114], ENCODE H3K27Ac mark [112], and dbSNP 150 [115] using the University of California at Santa Cruz (UCSC) database (http://genome.ucsc.edu/) [116]

of hypermethylation of the CpG island with tumor development [87]. Later studies confirmed the correlation between *RB1* hypermethylation and decreased *RB1* expression level [88]. Besides *RB1*, promoter methylation of other genes also affects retinoblastoma development. Our previous work found *MGMT* (10q26) promoter hypermethylation would lead to *MGMT* inactivation and to impairment of the functions of this enzyme in retinoblastoma [89, 90]. In 8 of 23 retinoblastoma samples, *MGMT* promoter hypermethylation was detected [89]. Similar observation was obtained in another cohort of 10 out of 68 samples [90]. O-6-methylguanine-DNA methyltransferase (MGMT) is an important enzyme in preventing DNA mismatch mutation. Under normal conditions, deoxycytidine (dC) is incorporated into the newly synthesized DNA strand opposite to deoxyguanosine (dG) during DNA replication. When there is alkylation on an oxygen atom (O^6) of dG, incorrect incorporation of thymidine (dT) would happen opposite to the O-6-methylguanine, which gives rise to DNA mismatch mutation and further compromises the genome integrity in retinoblastoma. MGMT converts O-6-methylguanine into dG to prevent this mismatch mutation. Another DNA mismatch repair protein *MLH1* (3q21.3) also showed promoter hypermethylation, which was associated with null MLH1 protein expression in retinoblastoma [91]. MLH1 coordinates the detection of the distorted DNA structure caused by the mismatch and removal of the mismatch DNA sequence [92]. Another example of promoter hypermethylated gene is *RASSF1A* (3q21.3). *RASSF1A* is a tumor-suppressor gene, which belongs to the RAS-association domain family. Its promoter was found hypermethylated in more than 50% of retinoblastoma samples, in 10 of 17 samples in a US cohort [92] and 56 of 68 samples in our Hong Kong Chinese cohort [86].

24.10.2 MicroRNA (miRNA)

MicroRNAs are small noncoding RNAs of 20–22 nucleotides that work as gene regulatory molecules by targeting mRNAs for degradation, resulting in translational repression [93]. It plays regulatory roles in the initiation and progression of human cancers [94]. Dysregulation by miRNA in retinoblastoma has been screened in microarray analyses [95–97]. The let-7 family members were involved in repressing oncogenes such as the RAS family members and MYC [95]. Another miRNA cluster miR17–92 regulates cell proliferation, differentiation, and angiogenesis. It was overexpressed in retinoblastoma tissues [96]. Furthermore, it was under direct regulation of *E2F* transcription factor. Therefore, in pRB-deficient cells, the activated E2F could further induce the expression of the miR17–92 cluster. Alternatively, the miR17–92 cluster could regulate the expression of E2F. Thus there is a regulatory feedback loop between the miR17–92 cluster and E2F [98, 99].

24.11 Genetic Counselling, Awareness Campaign, and Development of Novel Treatment

Retinoblastoma is a malignancy of early life, so early diagnosis and treatment are extremely important. Genetic counselling is an integral part of the management of retinoblastoma patients and their families. It is needed to assist parents in predicting the genetic features of retinoblastoma, which could be used to estimate the risk of having retinoblastoma in other family members. In Hong Kong, all children are screened for retinoblastoma at birth and at 5 years old, and the mean age of retinoblastoma presentation is 10 months after birth for bilateral cases and 26 months for unilateral cases. The advocate of early recognition of leukocoria with a smartphone flash photography by parents could effectively enhance the early identification of retinoblastoma. Recently we have launched a Hong Kong Retinoblastoma Awareness Campaign to promote early parental recognition of signs of retinoblastoma by linking with the citywide vaccination program for all newborn babies.

Current treatments of retinoblastoma mainly involve the application of chemotherapy, focal

treatment (laser or cryotherapy), or plaque radiotherapy [100, 101]. For advanced retinoblastoma, enucleation is unavoidable. It causes total loss of vision and imposes on the affected children psychological and mental trauma. The management of retinoblastoma aims to save the patients' life, reducing the risk of long-term secondary tumors, preserve globe, and maximize vision. Recent study found retinoblastoma affected mainly the cone precursor cells in the retina [102]. In the cone precursor cells, high levels of MDM2 were detected. MDM2 suppresses p53-mediated apoptosis. As *RB1* inactivation accelerates cell cycle, triggering apoptosis could stop the formation of cancer from these *RB1*-deficient cells. However, the lack of RB1 and high level of MDM2 in cone precursor cells work synergistically to initiate tumorigenesis. Accordingly, alterative pathways that can induce apoptosis in cone precursor cells might be effective in controlling the onset of tumor. Recently our group has found that inhibiting the receptor of a hypothalamic hormone, growth hormone-releasing hormone (GHRHR), could induce apoptosis specifically in retinoblastoma cells, without influencing other retinal cells [103]. GHRHR is highly expressed in retinoblastoma cell lines. Two antagonists of the GHRHR successfully suppressed the expressions of cell proliferation genes and induced the expressions of apoptotic genes in the retinoblastoma cell lines [103]. This study provides a promising approach to suppress tumor growth, with therapeutic potential for retinoblastoma.

24.12 Conclusive Remarks

There have been comprehensive studies reported in genes associated with retinoblastoma in different ethnic groups. Results of our studies in Chinese have led to new information on retinoblastoma development due to *RB1* gene variations and epigenetic changes of *RB1*, *MGMT*, *MLH1*, and *RASSF1A*. Subsequent studies in other populations produced consistent results. Our exploration of GHRHR antagonists in retinoblastoma cells gave indications of their potential therapeutic effects. Occurrence of retinoblastoma is pan-ethnic, and research conducted in different parts of the world has attributed to advancements of detection, treatment, and counselling of the disease, and more concerted international efforts are in view.

Compliance with Ethical Requirements Bi Ning Zhang, Yuning Jiang, Wai Kit Chu, Winnie W.Y. Lau, Simon T.C. Ko, Kwong Wai Choy, Calvin C.P. Pang, Guy L.J. Chen, and Jason C.S. Yam declare that they have no conflict of interest.

No human or animal studies were performed by the authors for this article.

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Genetics of Retinoblastoma: Basic Research and Clinical Applications

25

Usha Kim, K. Thirumalairaj, Aloysius Abraham, Shanthi Radhakrishnan, B. Devarajan, V. R. Muthukkaruppan, and A. Vanniarajan

Abstract

Retinoblastoma is the first genetic cancer of the eye, where the inheritance of the disease is directly demonstrated. This prototype cancer has served as a model for studying multiple other cancers. Retinoblastoma affects the vision, eye, and even life of young children up to the age of 5 years. Developing countries like India harbor the large number of retinoblastoma patients, and most of the patients present at advanced stage of the disease that makes the treatment difficult. It is hence proposed that early detection is necessary for the appropriate treatment and management of the disease. Basic research had largely helped the clinic to identify the patients with genetic predisposition to develop retinoblastoma. Effective use of genetic methods including next-generation sequencing had largely improved the patient care through high-throughput, large gene panel analysis and faster turnaround time. The newly adopted sequential genetic analysis method had considerably reduced the cost and provided an affordable method of genetic screening.

Keywords

Retinoblastoma · Genetic testing · Genetic counseling · Clinical applications · Gene panel · Pediatric cancer · Translational research · Genetics · Mutations · *RB1*

25.1 Introduction

Retinoblastoma (RB) (OMIM: 180200), an embryonal tumor of the developing retina, can affect early childhood from developing fetuses to 5-year-old children. It is the most common tumor of the eye contributing about 90% of ocular tumors and 4% of all tumors in young children

U. Kim
Department of Orbit, Oculoplasty and Ocular Oncology, Aravind Eye Hospital, Madurai, India

K. Thirumalairaj · A. Abraham · A. Vanniarajan (✉)
Department of Molecular Genetics, Aravind Medical Research Foundation, Madurai, India
e-mail: vanniarajan@aravind.org

R. Shanthi
Department of Pathology, Aravind Eye Hospital, Madurai, India

B. Devarajan
Department of Bioinformatics, Aravind Medical Research Foundation, Madurai, India

V. R. Muthukkaruppan
Advisor-Research, Aravind Medical Research Foundation, Madurai, India

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(NCRP-ICMR 1999–2000). There is an estimated incidence of 5000 new cases per year worldwide. The incidence of RB is even much higher with 1500–1800 new cases in developing countries like India compared to 280 new cases in the USA every year [1, 12]. This is primarily attributed to the increased population size and delayed presentation of the disease in the developing countries [1].

The delayed presentation is largely due to the socioeconomic factors such as lack of access to healthcare facilities, ignorance about early signs, illiteracy, poverty, lack of trained personnel, and inadequate infrastructure [2]. The globe salvage becomes difficult at the advanced stages, but the cure rate is higher with the surgical removal of the globe filled with tumor. If the tumor is detected early even before the gross appearance, it is possible to treat it completely, and vision can also be preserved. Genetic methods have proven to be useful in identifying the predisposed RB at least in patients having a positive family history. The various genetic studies in Indian RB patients and prospects of implementing them in the clinic are well documented in a recent review [1].

The outcome of the research has been witnessed in the recent years through the incorporation of the heritability factor for RB, and it is the first cancer where it has been widely accepted in clinical practice [3]. Earlier, the treatment of RB was only dependent on the clinical diagnosis. However, with the explosion of the molecular methods, the ophthalmologist is now armed with better ways of diagnosis and management. In many academic centers, the research labs are located in close vicinity to the clinics for improved communication between the clinician and geneticist. This also facilitates the patient to undergo the necessary tests for clinical and molecular diagnosis in a single visit. The clinical and genetic data are shared between the departments in order to provide the appropriate counseling to the patient and the family. This chapter will focus on the current updates in basic research in genetics and its usefulness in the diagnosis and management of RB.

25.2 Knudson's Hypothesis and Clinical Presentation of Retinoblastoma

Retinoblastoma is the first genetic cancer of the eye, where the inheritance of the disease is directly demonstrated and further research has helped to understand RB in detail (Table 25.1). RB may occur either as unilateral or bilateral

Table 25.1 From bed to bench and back to bed: a research path to RB1 genetic testing

1886: Retinoblastoma inheritance (Albert 1987)
First evidence of susceptibility to cancer inherited from a parent to a child by Brazilian ophthalmologist Hilário de Gouvêa. Two out of seven children born to a father who was successfully treated for childhood retinoblastoma also developed the disease.
1971: Two-hit hypothesis (Knudson 1971)
Alfred Knudson showed that two mutations are required for the occurrence of retinoblastoma by the analysis of 48 cases of retinoblastoma with laterality and the presence of a family history. In familial cases, one hit was inherited and the other one was acquired later; in sporadic tumors, both changes were somatic and are acquired during development.
1973: Tumor suppressor gene (Comings 1973)
David Comings articulated a general framework for a role of tumor suppressor genes. He explained that germline mutation in regulatory genes that suppressed tumorigenesis followed by the somatic loss of the homologous allele caused the inherited tumors including retinoblastoma.
1983: Localization of retinoblastoma gene (Cavenee et al. 1983)
Webster Cavanee and colleagues localized the retinoblastoma gene (*RB1*) to chromosome 13; Loss of heterozygosity was also demonstrated by the analysis of the inherited and sporadic cancers.
1986–1987: Isolation and cloning of *RB1* (Friend et al. 1986; Lee et al. 1987; Fung et al. 1987)
Stephen Friend and colleagues isolated a human cDNA that mapped to the *RB* region in 1986. The next year, *RB1 was* cloned by chromosome walking by two independent groups — Wen-Hwa Lee and Yuen-Kai Fung.
1989: Genetic testing of *RB1* (Yandell et al. 1989)
David Yandell and his peers had used DNA sequencing to identify point mutations in RB1 gene and reported that somatic mutations caused non-hereditary retinoblastoma, and germline mutations caused hereditary retinoblastoma.

depending upon the inheritance pattern. Bilateral RB is usually heritable caused either due to inherited or de novo germline mutations, and unilateral RB is usually nonheritable caused by somatic mutations. This difference in the inheritance pattern was explained by Knudson 46 years ago even before the *RB1* gene was described [4]. According to Knudson's hypothesis, mutation in both alleles of *RB1* gene was necessary for initiation of RB. The two-hit hypothesis of Knudson was further demonstrated in other pediatric tumors such as Wilms' tumor, neuroblastoma, and adult tumors including hemangioblastoma and renal cell carcinoma [5].

In case of bilateral RB, one mutation is already inherited, and hence the time taken for the occurrence of second mutation is less, and hence the patients with bilateral RB present early. In case of unilateral RB, both mutations occur in the retinal cells during the development, and RB may present late during the 2–3 years or after. Apart from those individuals who get RB, there were unaffected carriers who can transmit the mutant gene (without presenting with the disease). In these carriers, the second random mutation did not occur, and hence they did not get the disease [4].

Knudson's hypothesis, which was based on the statistical analysis of the laterality, focality, and family history of RB patients, was revisited recently in the Indian context with the genetic data.

In the study of 73 RB patients, 31 were bilateral, and 42 were unilateral. Multiple tumors were seen more common in bilateral group than unilateral group. The mean age at diagnosis for bilateral and unilateral cases was 9.82 ± 11.52 and 24.02 ± 15.11, respectively, and the mean difference in two groups was found to be statistically significant. In bilateral group, 21 (68%) cases presented at an age less than 1 year, while in unilateral group, most of the patients (18.43%) presented during the third year. Thus bilateral tumors presented earlier as compared to unilateral tumors. This study has reemphasized Knudson's hypothesis and correlated it with the age of the presentation [38].

25.3 Cost-Effective *RB1* Genetic Testing

RB1 is a large gene having 27 exons spread across 180 kb intervened by introns as long as 60kb. The promoter, exon 1 and several other exons have high GC content and repeat regions. Earlier, karyotyping and Southern blotting was used to detect whole *RB1* gene deletions [6, 7]**.** Point mutations were detected by single-strand conformation polymorphism and denaturing high performance liquid chromatography [8, 9], but with a low resolution. Later, multi-technique approaches were employed utilizing allele specific PCR, quantitative multiplex polymerase chain reaction (QM PCR) and Sanger sequencing to capture the complete spectrum of mutations [10, 11]. These multi-technique approaches remain time consuming, labour-intensive, and expensive.

In order to overcome all these bottlenecks, a new method with four simple steps based on the order of mutations as observed in the database and literature was reported [12]. Analysis of the database revealed that mutations are predominantly present in 8 exons having fragile codons leading to truncation. Based on the frequency of the pathogenic mutations (Fig. 25.1), a new strategy was suggested for *RB1* genetic screening. The first step involves Sanger sequencing of the selected exons, multiplex ligation dependent probe amplification (MLPA) in the step 2 followed by Sanger sequencing of other set of exons in step 3 and 4. The outcome of this study clearly matched with the published data in the order of nonsense mutations, deletions/duplications, splice variants and novel mutations [12].

A similar sequential mutation detection strategy was also reported in Tunisian RB patients in order to lower analytical efforts and reduce the cost of genetic testing. Out of 20 unrelated patients with familial and/or de novo bilateral RB, 19 patients had oncogenic mutations. Mutational mosaicism was found in one unilaterally affected father of a bilateral proband and incomplete penetrance was observed in two mothers. RNA analysis in a family with the same mutation showed an in-frame loss of exon 9. This

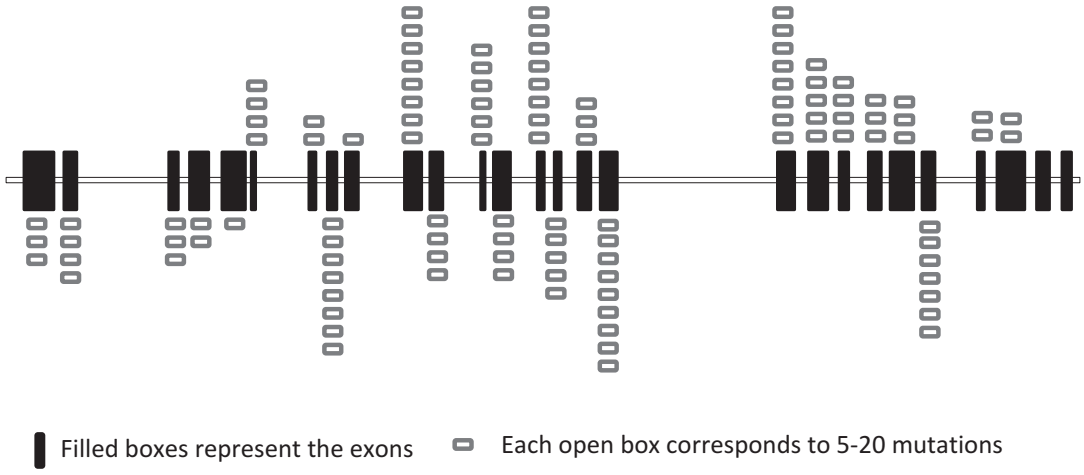

Fig. 25.1 Frequency of pathogenic mutation across the *RB1* gene

study emphasized the need for genetic testing to reveal or exclude incomplete penetrance specifically in parents of patients with sporadic disease [13].

25.4 Current Prospects of Next-Generation Sequencing

Since the spectrum of mutations identified in *RB1* gene varies widely, multiple methods are required for detecting different types of mutations. Sanger sequencing has been the choice for detection of point mutations and indels. Copy number changes including loss or gain of few exons to whole *RB1* gene can be detected by methods such as QMPCR, multiplex ligation-dependent probe amplification (MLPA), quantitative real-time PCR, and array comparative genomic hybridization (array CGH). With the technical advances, next-generation sequencing (NGS) provides the opportunity to find point mutations, indels, and copy number changes as well in a single run and make a direct impact on clinical management of RB.

NGS-based approaches provide a better way of genetic testing through enhanced sensitivity and faster turnaround and are cost-effective compared to the conventional methods. In a recent study of RB patients, in addition to the detection of other variants, amplification of *MYCN* was also included in a single panel, and whole assay is performed, and reports are generated in 3 days [14]. In another study of 50 unrelated patients with RB using the TruSight Cancer panel and run on a MiSeq platform, germline pathogenic mutations that included missense, nonsense, splice site, indel, and structural variants were identified in 66% (33/50) of the cases [15].

Another important application of NGS is the detection of low-level mosaicism. Sporadic RB is sometimes caused by de novo mutations at a threshold of 15–20%, which cannot be detected by Sanger sequencing. Through an ion-torrent-based deep sequencing, the frequency of point mutations in lymphocyte DNA was found to be increased from 96% to 97% for bilateral RB and from 13% to 18% for unilateral RB in a referral lab in the USA [16]. NGS could detect as little as 1% mutant through an artificial mosaicism created by serial dilution of mutants [17]. When a series of 30 patients with sporadic RB with no *RB1* mutations were retested, 3 had low-level mosaic variants, varying in frequency between 8 and 24% [18].

Targeted NGS had been shown to be a powerful tool in the molecular diagnosis of RB through the analysis of the RB patients from India. The algorithms used could detect both SNVs and CNVs [19]. Although NGS provides an advantage over the Sanger sequencing, still the algorithms used for detecting deletions and duplications are

evolving. Hence a combination of targeted NGS with array comparative genomic hybridization was suggested through an analysis of 65 RB patients and found a mutation detection rate of 96.5% in bilateral and 22% in unilateral [17].

Moreover, only the patient samples were analyzed in many studies, and interpretation was made based on the variants identified in the patient samples. However, in some cases, the variants identified might be benign without being cause of the disease. It is important to include the family members along with patients in order to confirm the variants as pathogenic through co-segregation analysis and classify them as inherited or de novo [19]. The enhanced ability of mutation detection by NGS has significant implications for improved clinical diagnosis, genetic counseling, surveillance, and management of RB.

25.5 Current Research Focused toward Clinical Applications

It is now understood that the biallelic inactivation of *RB1* is important for the development of RB tumor. The loss of two *RB1* alleles in turn will trigger the other changes leading to genomic instabilities leading to copy number gains in genes, such as *MDM4*, *KIF14*, *MYCN*, *DEK*, and *E2F*, and loss of *CDH11* [20]. It has been reported that a small proportion of RB can be caused by the copy number gains of *MYCN* alone even without the inactivation of the *RB1* gene [21]. Customized NGS panels are now under development to target the genes that are involved in the tumor progression.

Differential gene regulation of various cancer pathways and DNA damage pathways had been found in RB [22, 23]. It is proposed that many of these genes are regulated by epigenetic rather than genetic mechanisms as described in syk. Further assays with inhibitors of these genes showed promising results for tumor reduction and could be the potential drugs for RB patients. Further reduction of the tumor through inhibitors suggests the newer therapeutic drugs for RB patients [24, 25]. In another study, genes such as *MSH6*, *CD44*, *PAX5*, *GATA5*, *TP53*, *VHL*, and *GSTP1* along with the recurrently methylated *MGMT*, *RB1*, and *CDKN2* were found to be hypermethylated in RB tumors [26].

MicroRNAs (miRNAs) are single-stranded noncoding RNA molecules that contribute to posttranscriptional downregulation of gene expression. It is noted that microRNA-21 possesses the oncogenic potential to target several tumor suppressor genes, including *PDCD4*, and thereby downregulates *RB1* expression and regulates tumor progression and metastasis [27]. Inactivation of miR-17-92 suppresses RB formation in mice, and co-silencing of miR-17/20a and p53 cooperatively decreases the viability of human RB cells [28]. In a miRNome landscape analysis, core cluster of 30 miRNAs were found to be highly expressed in RB tumors [29]. Recent studies focus on studying the levels of miRNAs in body fluids which may serve as a biomarker for RB.

25.6 Implications of Genetic Testing

Genetic testing of *RB1* gene has made a significant impact in the clinical management by (i) predicting the risk, (ii) assessing the carrier status, (iii) confirming the diagnosis, and (iv) prenatal or preimplantation diagnosis. Based on the mutation and its inheritance pattern, classification of retinoblastoma into heritable and nonheritable is possible (Table 25.2).

Table 25.2 Clinical implications of heritable and nonheritable retinoblastoma

Heritable retinoblastoma	Nonheritable retinoblastoma
Generally detected early (0–2 years)	Generally detected late (3–5 years)
Mostly bilateral	Mostly unilateral
Mostly multifocal	Mostly unifocal
Either inherited or de novo	Not inherited
Increased risk of secondary tumors	No risk of secondary tumors
Higher risk of RB in sibling and offspring	Low risk of RB in sibling and offspring

25.6.1 Predicting the Risk

Genetic testing of *RB1* will facilitate the risk prediction in the siblings and offspring of the affected individuals [30]. (a) Close surveillance of the sibling/offspring is required if the inherited germline mutation is detected as the risk is estimated to be 50%. (c) If the mutation is found to be de novo germline, the risk will reduce to 5% in the sibling possibly due to gonadal mosaicism of one of the parents and 50% risk in the offspring. (d) If the mutation is found to be somatic, the risk of RB reduces to less than 1% in sibling and offspring which is equal to the general population risk.

If the mutation is identified in a family having one of the parents and a child affected with RB, then the next sibling is at a higher chance of developing RB. In this case, early screening of the sibling soon after birth will help in surveillance of the disease in the child. Early diagnosis through the genetic testing will help in providing the appropriate treatment to save the eye and vision. If the heritable mutation in the family is not identified in the child examined after birth, further surveillance may not be necessary and could avoid unnecessary examinations under anesthesia. The patients with *RB1* mutation will have an increased risk of developing pinealoblastoma and lifelong risk of developing secondary tumors, and hence periodical surveillance is necessary.

25.6.2 Assessing the Carriers of the RB1 Mutations

Rarely, there may be carriers of the *RB1* mutations without any disease phenotype, either due to the nonoccurrence of the second mutation in the retinal cells or spontaneous regression of the tumor. However, they can potentially pass on the mutation to the next generation. It is hence important to screen the complete family when a mutation is identified in the patient for the accurate risk prediction for the next sibling.

25.6.3 Confirmation of Diagnosis

Genetic testing of *RB1* may also serve as a tool for confirming the clinical diagnosis. The predominant sign of the RB such as leucocoria often mimics other eye conditions including but not limited to persistent hyperplastic primary vitreous (PHPV), Coats' disease, ocular toxocariasis, and retinopathy of prematurity. The eye will be painful in most of these cases similar to RB and end up in enucleation. Genetic testing of *RB1* in most of these tissues obtained from enucleated globes will help in confirming the diagnosis of RB along with the histopathological analysis, thereby excluding the other etiologies.

This is important for further treatment protocols as chemotherapeutic drugs were administered even after enucleation if the extraocular spread was noticed by histological analysis. Some patients present with unilateral RB without any family history but with a germline mutation detected in constitutional cells. These patients may turn to bilateral within few months, and hence a close surveillance will help in saving the other eye and vision which otherwise may become worsen.

25.6.4 Prenatal or Preimplantation Genetic Diagnosis

The survival of the RB patients has considered increased with improved treatment procedures. Many adult patients who wanted to plan their family were anxious to know whether they would inherit the disease and like to know the possible ways of avoiding the children with RB. In case of patients having germline mutations, there would be an increased risk of inheriting RB to the child, and hence a prenatal genetic testing would help in identifying the risk of RB in the growing fetus.

If mutation was detected in fetal DNA, the pregnancy could be terminated to avoid the child with RB or continued further. If the pregnancy was continued, the screening of the child immediately after birth is required to check for RB and

further treatment. If the mutation was not detected in the fetal DNA, a chance of the inheritance would be reduced to less than <1, and a normal child was expected. Preimplantation genetic testing might also help to avoid the embryos carrying mutations that can potentially cause RB. After testing the embryos, the transfer of embryos without the mutation would produce an offspring free of RB.

25.7 Summary

It is now clear that basic research in retinoblastoma genetics has paved a way for the translational research and supported the clinical care through early detection and intervention of retinoblastoma. The enhanced sensitivity of mutation detection by NGS has significant implications for improved clinical diagnosis, genetic counseling, surveillance, and management of RB. The cost of the analysis has been considerably reduced by adapting newer strategies of genetic testing. In the developing countries like India, it is important to utilize the genetic methods for early detection of the disease which would reduce the economic burden and social stress due to the loss of vision and the eye.

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Genotype-Phenotype Correlation in Retinal Degenerations

26

Sripriya Srivatsan, Mathavan Sinnakaruppan, Vikas Khetan, Sundaram Natarajan, Sangeetha Srinivasan, and Rajiv Raman

Abstract

Vitreoretinal degenerations affect a significant proportion of people world-wide, which, in the most severe stages, can result in legal blindness. Investigations on molecular, genetic and environmental basis of these degenerations have contributed to additional knowledge with respect to differential diagnosis according to the genotype and gene therapy. This chapter summarizes the phenotype and genotype of the vitreoretinal degenerations under four major groups: (1) rod-dominated diseases, (2) cone-dominated diseases, (3) generalised retinal degenerations (affecting both photoreceptor cell types, rods and cones) and finally (iv) exudative as well as erosive vitreoretinopathies.

Keywords

Genotype · Phenotype · Retina · Degeneration

S. Srivatsan · M. Sinnakaruppan
SNONGC Department of Genetics and Molecular biology, Vision Research Foundation, Chennai, India

V. Khetan · S. Srinivasan · R. Raman (✉)
Shri Bhagwan Mahavir Vitreoretinal Services, Sankara Nethralaya, Chennai, India
e-mail: drrrn@snmail.org

S. Natarajan
Aditya Jyot Eye Hospital, Mumbai, India

26.1 Introduction

Monogenic diseases of the retina and vitreous affect approximately 1 in 2000 individuals or more than 2 million people worldwide [1]. Clinical features can range from legal blindness in the most severe forms of retinal degenerations (Leber congenital amaurosis, LCA) to less severe or rather mild retinal dysfunctions (night blindness, achromatopsia). In the past two decades, the knowledge about the molecular basis of retinal diseases has tremendously progressed, and evidence for the contribution of genetic factors but also environmental circumstances is continuously accumulating. However, with the available techniques, a reliable molecular diagnosis is possible for only half of the affected individuals or families with monogenic forms of retinal diseases [2]. In addition, the predictive value of a mutation or risk allele for multifactorial disorders is problematic since the phenotypic and/or symptomatic consequences are highly variable. Nevertheless, this knowledge has greatly improved the understanding of molecular aetiology to some extent. The diseases discussed in this article are categorised in four major groups: (1) rod-dominated diseases, (2) cone-dominated diseases, (3) generalised retinal degenerations (affecting both photoreceptor cell types, rods and cones) and finally (iv) exudative as well as erosive vitreoretinopathies.

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Each disorder is further described as genotype and phenotype to understand the genotype-phenotype correlation to some extent.

26.2 Non-syndromic Retinal and Vitreoretinal Diseases

26.2.1 Disease of Rod Photoreceptor Cells (Stationary and Progressive)

26.2.1.1 Stationary Rod Diseases

Congenital Stationary Night Blindness

Phenotype As the name implies, congenital stationary night blindness (CSNB) is a nonprogressive visual impairment present at birth. It is a rod degenerative disease leading to characteristic features of night vision impairment and decreased visual acuity. CSNB is classified according to ERG findings, where in Schubert-Bornschein type, the b wave is smaller than the a wave and Riggs type is defined by proportionally reduced a and b waves [3].

Genotype Genetically it shows autosomal dominant, recessive and X-linked recessive pattern of inheritance.

Riggs–Type CSNB Autosomal dominant genes involved in Riggs-type CSNB are *RHO*, *GNAT* and *PDE6B*, while only one gene *SLC24A1* is inherited recessively.

Schubert-Bornschein–Type CSNB It is subdivided as complete and incomplete according to ERG. The complete form has ON-bipolar cell dysfunction and hence characterised by reduced rod b-wave response. The autosomal recessive genes in complete CSNB are *GRM6*, *GNB3*, *TRPM1*, *GPR179* and *LRIT3*. *NYX* is inherited as X-linked recessive form.

Incomplete CSNB involves both ON- and OFF-bipolar cell dysfunction caused due to genes involved presynaptically and in glutamate release. The X-linked incomplete CSNB is associated with mutation in *CACNA1F*, while the autosomal recessive CSNB is associated with mutations in *CABP4*, *CACNA2D4*, *GNAT1* and *SLC24A1* [3].

Oguchi Disease

Phenotype Oguchi disease is an autosomal recessive stationary rod disease. It is differentiated from CSNB by the characteristic fundus change where golden yellow fundus reflex was observed which returns to its normal appearance after long dark adaptation.

Genotype Genetically also it does not overlap with CSNB where rod phototransduction genes like arrestin gene (s-antigen, *SAG*) and rhodopsin kinase gene (G protein-coupled receptor kinase 1, *GRK1*) are shown to be causative in many patients.

Fundus Albipunctatus

Phenotype Fundus albipunctatus is an autosomal recessive congenital night blindness differentiated from CSNB due to fundus involvement. Also it is differentially diagnosed from Oguchi by phenotype difference, i.e. presence of white to white-yellow dots in the perimacular or periphery of the retina.

Genotype The genes so far associated with this disease are *RDH5, RLBP1* and *RPE65* [4].

26.2.1.2 Progressive Rod Diseases

Retinitis Pigmentosa (RP)

Phenotype RP is a heterogeneously inherited progressive retinal degeneration with characteristic symptoms of night blindness, tunnel vision and progressive decrease in central vision. Retinal manifestations include narrowing of arterioles, pigmentation within the retina combined with loss of pigmentation and retinal pigment epithelial cells resulting in a bone-spicule presentation. More advanced stages present with cataract, pale optic disc and severe narrowing of the arterioles. The condition is generally bilateral but can present with no detectable fundus findings but with degeneration of the outer retinal layers (also called RP sine pigmento) to sectoral or diffuse retinal

involvement. To assess the disease status and progression, electroretinographic measurements provide a sensitive method. In contrast to CSNB, which predominantly affects the b wave, RP is characterised by reduced or absent a and b waves in the ERG.

Juvenile RP or Early–Onset RP

Phenotype Juvenile RP is always confused with Leber congenital amaurosis as it affects rod and cone photoreceptors simultaneously. While LCA is defined as blindness earlier to 1 year of age, EORP is considered in those kids with progressive blindness later than 1 year.

Genotype The autosomal recessive form has mutation in *SPATA7*, *LRAT* and *TULP1*, and autosomal dominant form has mutation in AIPL1 gene.

Late–Onset RP

Genotype The onset is from the second decade onwards and is inherited as autosomal dominant/recessive, X-linked and also y-linked. Autosomal recessive inheritance is more frequently reported with mutations in ABCA4, AGBL5, *ARHGEF18*, *ARL6*, *ARL2BP*, *BBS1*, *BBS2*, *BEST1*, *C2orf71*, *C8orf37*, *CERKL*, *CLRN1*, *CNGA1*, *CNGB1*, CRB1, CYP4V2, *DHDDS*, *DHX38*, *EMC1*, *EYS*, *FAM161A*, *GPR125*, *HGSNAT*, *IDH3B*, IFT140, *IFT172*, *IMPG2*, *KIAA1549*, *KIZ*, LRAT, *MAK*, MERTK, *MVK, NEK2, NEUROD1, NR2E3*, *NRL*, *PDE6A*, PDE6B, *PDE6G*, *POMGNT1*, *PRCD*, *PROM1, RBP3, REEP6, RGR, RHO, RLBP1, RP1, RP1L1, RPE65, SAG, SAMD11*, *TRNT1*, *TTC8*, TULP1, *USH2A*, *ZNF408* and *ZNF513*.The recessive loci mapped so far are 16p12 (RP22), 4q32-q34 (RP29) and 1p21.1-p13.3 (RP32).

The autosomal dominant RP account to about 30% of cases and are caused by *ADIPOR1, ARL3, BEST1, CA4, CRX, FSCN2, GUCA1B, HK1, IMPDH1, KLHL7, NR2E3,NRL, PRPF3, PRPF4, PRPF6, PRPF8, PRPF31, PRPH2, RDH12, RHO, ROM1, RP1, RP9,RPE65, SEMA4A, SNRNP200, SPP2 and TOPORS*. The dominant locus associated is 6q23 (RP63).

Only three genes have been associated with X-linked RP, *OFD1, RP2* and *RPGR*. Of this, *RPGR* is more frequently associated, and it is also shown to be involved in syndromic/atypical RP like in primary ciliary dyskinesias. The loci associated so far are Xp21.3-p21.2 (RP6), Xq26-q27 (RP24) and Xq28 (RP34). Y-linked inheritance is reported in one Chinese family [5].

Pericentral RP is defined as one which affects only the near-central retina and spared the periphery. Mutation studies in these patients have shown mutations in *RHO, USH2A,* PDE6B and *HGSNAT* genes.

Many syndromes have associated RP – Alstrom syndrome include abetalipoproteinaemia, Refsum syndrome, Bardet-Biedl syndrome, Usher syndrome, etc.

RHO is among the most prominent RP-associated genes. *RHO* constitutes a seven-transmembrane receptor protein which initiates the phototransduction cascade upon absorption of light by its chromophore 11-cis retinal. The vast majority of mutations show a classical autosomal dominant inheritance leading to RP, the mechanism of which is fairly well understood as being either a gain-of-function or a dominant negative effect of the mutated protein.

26.2.2 Cone and Cone-Rod Diseases (Stationary and Progressive)

26.2.2.1 Stationary Cone Dysfunction/Colour Vision Defect

The stationary cone dysfunction group of diseases are characterised by colour vision abnormalities, photophobia and electro- or psychophysical evidence of abnormal functioning of cone. They are clinically and genetically heterogeneous. The stationary cone dystrophies are mostly congenital, and hence they usually display a normal rod response.

Achromatopsia Achromatopsia are autosomal recessive cone diseases that are classified as i) a more severe form called complete achromatopsia

where all three types of cones are affected and ii) a less severe form called incomplete achromatopsia where patients have high visual acuity and mild cone response in ERG. There are five genes associated with the disease – *CNGA3*, *CNGB3*, *GNAT2*, *PDE6H*, *PDE6C* and *ATF6* [6]. All these genes are shown to be a part of phototransduction pathway in cone cells. Of these, incomplete achromatopsia involves mutation in *CNGA3* and *PDE6H* only [7, 8].

Oligoconetrichromacy Oligoconetrichromacy is a very rare cone disease which projects reduced visual acuity and cone response in ERG while a normal colour vision and retinal phenotype. It has a genetic overlap with genes of achromatopsia with association of recessive mutation in *CNGA3*, *CNGB3*, *GNAT2* and *PDE6C*.

Blue Cone Monochromatism (BCM) It is also a rare disease of cone dysfunction characterised by severe impairment in colour discrimination, photophobia and reduced visual acuity. It is also sometimes referred as a form of incomplete or atypical achromatopsia. But the distinguishing factor is the loss of function mutation in *OPN1LW* and *OPN1MW* genes encoding 'L' (red) and 'M' (green) cone pigments. These genes are present in X chromosomes, and hence these diseases are X-linked recessive.

26.2.2.2 Cone and Cone-Rod Dystrophies (COD and CORD)

This involves cone degeneration in childhood or early adult stage and subsequent degeneration of rods later. Genetically it is highly heterogeneous with overlapping genotype to many retinal degenerative diseases like RP, LCA, macular degeneration, rod dystrophies, etc.

Phenotype The progressive cone diseases are generally more severe than the progressive rod-dominated phenotypes (e.g. RP). Although some peripheral vision is preserved, it can lead to legal blindness earlier than RP. Affected individuals experience first symptoms of decreased visual acuity at school during the first decade of life. In addition, patients with progressive CODs and CORDs feel intense photophobia and variable degrees of colour vision abnormalities. Central scotomas can be also present upon visual field testing. The fundus examination frequently reveals pigment deposits and retinal atrophy in the macular region. To discriminate CORDs from CODs and macular degeneration, additional ophthalmologic examinations are needed (fluorescein angiography, fundus autofluorescence, ERG). In contrast to CODs, CORDs show a peripheral retinal involvement, and the ERG is characterised by a decrease in both cone and rod responses. However, cone responses are more severely affected than the rod-specific ERG components. In pure CODs or early CORDs, scotopic ERG is normal. In later disease stages, night blindness occurs, and the loss of the peripheral visual field is progressing. This is different in rod-dominated retinal diseases, where night blindness is one of the first symptoms and the disease progresses from the periphery to the centre.

Genotype It is inherited in autosomal dominant, recessive and X-linked recessive manner. The genes involve in autosomal dominant CORDs are *AIPL1*, *CRX*, *GUCA1A*, *GUCY2D*, *PITPNM3*, *PROM1*, *PRPH2*, *RIMS1*, *SEMA4A* and *UNC119*.The loci involved are 17q and 10q26.

The autosomal recessive CORD genes include *ABCA4*, *ADAM9*, *RPGRIP1*, *CDHR1*, *C8orf37*, *RAB28*, *TTLL5*, *POC1B*, *ATF6*, *C21orf2*, *CACNA2D4*, *CERKL*, *CNNM4*, *IFT81*, *KCNV2*, *RAX2* and *RDH5*.The unidentified gene locus involved is 1q12-q24. The X-linked recessive progressive CORD is associated with mutation in RPGR, *CACNA1F* genes and Xq27 loci.

26.2.3 Macular Degeneration

26.2.3.1 Monogenic

Best Disease Best disease, also called as Best vitelliform dystrophy, is an early-onset macular dystrophy caused by mutation in *BEST1* (VMD2) gene. This gene encodes bestrophin-1 that functions as a calcium chloride channel. It is inherited as an autosomal dominant disease. Incomplete

penetrance is also noted in few cases [9]. There is also a separate class of bestrophinopathy called the autosomal recessive bestrophinopathy (ARB) caused by recessive mutation in *BEST1* gene [10].

Sorsby's Fundus Dystrophy Sorsby's fundus dystrophy is an age-related autosomal dominant macular dystrophy occurring in the fourth decade of life [11]. Mutation in tissue inhibitor of metalloproteinase 3 (*TIMP3*) gene is shown to be associated with the disease [9].

Malattia Leventinese and Doyne Honeycomb Retinal Dystrophy Malattia leventinese (ML) and Doyne honeycomb retinal dystrophy (DHRD) are autosomal dominant diseases that have a very strong phenotypic similarity to age-related macular degeneration (AMD). Unlike AMD, they are monogenic where mutations in *EFEMP1* (EGF-containing fibrillin-like extracellular matrix protein 1) gene are shown to be associated with it [12].

X-Linked Juvenile Retinoschisis Retinoschisis is an X-linked recessive disorder caused by mutation in *RS1* gene encoding retinoschisin [13]. There is also a study of single three-generation family with eight affected members showing typical autosomal dominant pattern of the disease [14].

26.2.3.2 Multigenic

Adult-Onset Vitelliform Macular dystrophy (AVMD) Vitelliform macular dystrophies (VMDs) are characterised by round yellow deposits at the centre of the macula and contain lipofuscin deposits in the fundus autofluorescence.

AVMD are late-onset autosomal dominant genetically heterogeneous macular dystrophies caused by mutation in *PRPH2* (VMD3), *IMPG1* (VMD4) and I*MPG2* (VMD5) [15].

Stargardt's Disease (STGD) It is the common juvenile macular degeneration with clinical and genetic heterogeneity. So far three genes are associated with it – *ABCA4* [16], *PROM1* and *ELOV4*. *PROM1* [17] is inherited in autosomal recessive manner, while *ELOVL4* [18] is found to be mutated in autosomal dominant families.

Fundus flavimaculatus (FFM) is an allelic disorder of Stargardt's disease with mutations in *ABCA4* and *PRPH2* [19]. It is characterised as a late-onset form of Stargardt's disease.

Age–Related Macular Degeneration (AMD) ARMD is a multifactorial disorder with associated genetic and environmental factors. ARMD is classified as dry and wet AMD depending on the presence or absence of drusen. It is autosomal dominant and is also genetically heterogeneous with association of high-risk polymorphism in genes like *FBLN6*, *CFH*, *VEGF*, *LRP6*, *HTRA1*, *HLA*, *MMP9*, *ARMS2*, *C3* and *TLR4* and risk variants in *ABCR*, *FBLN5*, *ERCC6*, *ELOVL4*, *APOE*, *ACE*, *SOD2*, *ABCA4*, *PON1*, *RAX2*, *CST3*, *CX3CR1*, *CFI*, *C9*, *C2* and *CFB*. AMD is also associated with mitochondrial gene mutation in *MTTL1* [20]. About 50% of the disease risk is attributed to *CFH* polymorphism Y402H [21, 22]. There are also reports that show that the combination of environmental factors like smoking and body mass index along with Y402H variation increases the risk of AMD [23]. Reduced risk of AMD has been associated with common deletion that encompasses both the *CFHR1* and *CFHR3* genes [24].

26.2.4 Generalised Photoreceptor Diseases

26.2.4.1 Leber Congenital Amaurosis

Phenotype Leber congenital amaurosis is a severe form of retinal degenerative disease diagnosed in children earlier than 1 year of age. This disease was described initially by Theodore Leber in 1869 as a congenital form of retinitis pigmentosa [25].The clinically distinguishing features of LCA include severe visual impairment present at birth or shortly thereafter, extinguished or non-recordable ERG, pendular or searching nystagmus, photophobia and digito-ocular sign (Franceschetti-Leber phenomenon), with progressive retinal degeneration [26].

Genotype LCA is both clinically and genetically heterogeneous. So far, 29 candidate genes have been identified. They are *GUCY2D (LCA1)*, *RPE65 (LCA2), SPATA7(LCA3)*, *AIPL1(LCA4)*, *LCA5(LCA5)*, *RPGRIP1(LCA6)*, *CRX (LCA7)*, *CRB1(LCA8)*, *NMNAT1(LCA9)*, *CEP290 (LCA10)*, *IMPDH1(LCA11)*, *RD3 (LCA12)*, *RDH12 (LCA13)*, *LRAT(LCA14)*, *TULP1 (LCA15)*, *KCNJ13 (LCA16)*, *GDF6 (LCA17)*, *PRPH2 (LCA18)*, *CNGA3*, *CLUAP1*, *DTHD1*, *IQCB1*, , *MERTK*, *MYO7A*, *OTX2*, *ALMS1*, *CABP4* and *CCT2*. Most of them are inherited in an autosomal recessive manner except *CRX*, *IMPDH1* and *OTX2* which are associated with autosomal dominant inheritance pattern [27, 28].

One of the genes which is associated with LCA was designated *RPE65* [29]. It encodes a protein consisting of 533 amino acid residues with high abundance in the retinal pigment epithelium. The protein is an isomerase and involved in the conversion of all-trans retinol to 11-cis retinal. Gene transfer of the *RPE65* gene in the eyes of patients provides the first example for a successful gene therapy in human patient with this severe form of retinal dystrophy [30–32]. LCA like ocular phenotype is also observed in few syndromes like Alstrom syndrome (*ALMS1* gene mutation), infantile neuronal ceroid lipofuscinoses, Senior-Loken syndrome (*IQCB1* gene mutation), Joubert syndrome (*CEP290* gene mutation) and thiamine-responsive megaloblastic anaemia (*SLC19A2* gene mutation).

26.2.4.2 Choroideremia

Phenotype Choroideremia is an X-linked dominant chorioretinal dystrophy. Symptoms begin with night blindness during teenage years, and the disease progressively affects degeneration of photoreceptor cells, retinal pigment epithelial cells, choriocapillaris and choroid, leading to complete blindness.

Genotype Choroideremia is caused by mutation in *CHM* gene. CHM encodes Rab escort protein 1 (*REP1*), a family of GTP-binding proteins that regulate vesicular traffic. Absence of *REP1* leads to degeneration of choroid, photoreceptors and retinal pigment epithelial cells leading to severe blindness [33]. In contrast to mutations that affect only the *REP1* locus leading to non-syndromic CHM, large gene deletions are known to cause syndromic phenotypes [34].

The initial phase of an ongoing multicentric clinical trial reported promising results. Six patients were administered a subfoveal injection of adeno-associated viral (AAV) vector-encoding *REP1*. There was a significant improvement in visual acuity and in rod and cone functions [35].

26.2.4.3 Gyrate Atrophy of the Choroid and Retina

Phenotype Gyrate atrophy of the choroid and retina is a progressive condition associated with significantly increased plasma ornithine levels, chorioretinal degeneration, myopia and early cataracts. There might be a mild skeletal muscle weakness and mental retardation, but these extraocular symptoms are present only occasionally.

Genotype It is inherited as an autosomal recessive disease and is shown to be caused by homozygous or compound heterozygous mutation in the ornithine aminotransferase (*OAT*) gene [36].

26.2.5 Vitreoretinopathies

26.2.5.1 Erosive Vitreoretinopathies (ERVR)

Phenotype Erosive vitreoretinopathy belongs to a group of disorders called the hereditary vitreoretinopathies [37]. Apart from erosive vitreoretinopathy, Stickler syndrome, Wagner's disease and Goldmann-Favre syndrome also belong to hereditary vitreoretinopathies and are characterised by marked vitreous syneresis. The differential diagnosis for ERVR phenotypically involves marked visual field defect, poor night vision, abnormal electroretinographic findings and associated retinal pigment epithelial changes [38]. This along with absence of *COL2A1* gene muta-

tion helps in differential diagnosis of ERVR and Stickler syndrome.

In most of the patients, the first clinical sign becomes obvious during the late teens. The effected vitreous is frequently described as 'empty' with veils and strands. Except for autosomal dominant vitreoretinochoroidopathy, retinal detachment is a common feature in all. Wagner's and erosive vitreoretinopathy present with tractional retinal detachment/snowflake degeneration, while Stickler syndrome presents with rhegmatogenous retinal detachment.

Genotype Genotypically ERVR is an autosomal dominant disease and is considered to be an allelic disorder of Wagner's disease. Brown et al. [39] observed a significant linkage of both ERVR and Wagner's disease to regions on chromosome 5q13–14 [39]. On screening the critical region using 13 microsatellite markers, a *CSPG2*/*Versican* splice site variant was observed in families of ERVR and Wagner's disease. *CSPG2*/Versican functions to maintain the integrity of vitreous by keeping the collagen molecules apart. The splice variant results in the balance shifts of the *CSPG2*/*Versican* isoforms [40], thus leading to disease phenotype.

Selective Phenotypes of Few ERVRs

Stickler Syndrome (STL)

Phenotype Stickler syndrome was initially designated as hereditary progressive arthro-ophthalmopathy, a connective tissue disorder involving skeletal, orofacial, ocular and auditory abnormalities [41]. Along with the characteristic vitreous syneresis observed in all hereditary vitreoretinopathies, various other abnormalities reported with this syndrome includes mild spondyloepiphyseal dysplasia or arthritis, cleft palate, both conductive and sensorineural hearing loss and ocular findings such as retinal detachment, high myopia and cataract [42]. The prevalence of Stickler syndrome is 1 in 10,000 newborns [43].

Genotype It is genetically heterogeneous with six candidate genes identified so far. The autosomal dominant STL includes mutations in *COL2A1* gene (STL type I) [44], *COL11A1* gene (STL type II) [45] and *COL11A2* gene (STL type III) [46]. Among these STL type III is a nonocular Stickler syndrome. Incomplete penetrance was also observed in a family with *COL2A1* dominant mutation resulting in variable age of onset [47].

The autosomal recessive STL includes mutations in *COL9A1* (STL type IV) [44] and *COL9A2* genes (STL type V) [48, 49]. Also, autozygome and exome analysis in a family identified a novel missense variant in *LOXL3* as the likely candidate cause [50]. Mutation in these genes affects the fibrillar type II/XI collagen molecules expressed in vitreous interfering in the proper formation of secondary vitreous.

Enhanced S–Cone Syndrome (ESCS)

ESCS patients have an unusual gain of function of photoreceptors with varying degree of severity of retinal degeneration. The more severe type is the Goldmann-Favre syndrome (GFS). GFS is characterised by a liquefied vitreous body with preretinal band-shaped structures, cataract, macular retinoschisis, pigment loss and severely affected ERG. The patients have severely reduced number of rods and L and M types of cones but increased S cones.

Phenotype The association of nummular pigmentary deposits with white-yellow dots at the level of the RPE along the vascular arcades, focal hyperpigmentation within the arcades and foveal or peripheral schisis is clinically suggestive of ESCS. The ERG features are the rod-specific undetectable ERG; the ERG to a standard flash is simplified and delayed, with a similar waveform under photopic and scotopic conditions; and, also of importance, the 30-Hz flicker is delayed and of lower amplitude than the single-flash photopic ERG a wave. Further, abnormally large, delayed, simplified waveform S-cone ERG responses (relative to the size of the conventional ERGs) are present in majority of patients.

Genotype The molecular cause is related to mutations of nuclear receptor gene (*NR2E3*).

26.2.5.2 Exudative Vitreoretinopathy (EVR)

Exudative vitreoretinopathy is characterised by heterogeneous retinal vascular changes that vary from being avascular to developing retinal neovascularisation, vitreoretinal traction, subretinal exudation and retinal detachment.

Phenotype EVR is characterised by an incomplete blood vessel development in the retinal periphery and retinal folds or retinal detachment. The clinical diagnosis is usually made in the first years of life. Upon fluorescein angiography, the retinal periphery appears avascular. A fibrovascular mass may develop that is associated with retinal exudates. This mass may extend to the ciliary body and peripheral lens capsule. The clinical manifestations are highly variable, and some patients do not experience marked visual impairment.

Genotype In most cases, it is considered familial and is shown to inherit in autosomal dominant, autosomal recessive and X-linked recessive form. Genes that are associated with dominant form of the disease are Wnt signalling pathway proteins such as *FZD4* [51] on 11q14 encoding the putative Wnt receptor frizzled-4, *LRP5* gene on 11q13.4 encoding low-density lipoprotein receptor-related protein and cadherin-associated protein, beta1 *(CTNNB1)* [52] on chromosome 3p22. Other genes include tetraspanin family of protein encoded by *TSPAN12* [53] on 7q31 that functions with the norrin receptor complex and increases norrin/beta-catenin signalling, *ZNF408* [54], a transcription factor that functions in vascular development. While *LRP5* gene mutation was also associated with autosomal recessive EVR [55], X-linked recessive EVR is caused by mutation in *NDP* gene on chromosome Xp11 encoding norrin protein which is involved in a pathway that regulates neural cell differentiation and proliferation [56].

26.3 Syndromic Retinal Diseases

26.3.1 Usher Syndrome (USH)

Phenotype Clinically, Usher syndrome is classified in three subtypes, depending on the severity and age of onset of disease manifestations in the retina and ear. The most severe form is Usher syndrome type I. Patients with this subtype have profound and congenital deafness and vestibular dysfunction, leading to delayed motor development and adolescent-onset RP. Usher type II is less severe with normal vestibular function; mild to severe sensorineural hearing loss, which is nonprogressive in most cases; and a later onset of RP in adolescence or adulthood. Patients with Usher III also have a milder but progressive form of deafness, and approximately 50% also manifest vestibular problems.

Genotype Genes associated with USH type I are *MYO7A* (USH1B), Harmonin (USH1C), *CDH23* (USH1D), *PCDH15* (USH1F), *SANS* (USH1G) and *CIB2* (USH1J). Three loci associated with USH1 are 21q21 (USH1E), 15q22-q23 (USH1H), 10p11.21-q21.1(USH1K). USH type II genes include Usherin (USH2A), *GPR98* (USH2C) and *DFNB31* (USH2D), and USH type III genes are *CLRN1* and *HARS* (USH3A).

Digenic inheritance was also reported in few cases – atypical Ushers was reported with the combination of mutant homozygous *CEP250* and heterozygous *C2orf71* [57], in USH3 patient with homozygous *CLRN1* mutation and heterozygous *MYO7A* mutation [58]. With ubiquitous gene expression, the USH genes mostly function as cell adhesion proteins and scaffolds, in actin-based intracellular trafficking and Ca^{2+}-mediated signalling [59].

26.3.2 Bardet-Biedl Syndrome (BBS)

Phenotype Bardet-Biedl syndrome is a ciliopathy disorder caused by disruption in ciliary biogenesis and trafficking. It is clinically

heterogeneous and characterised by primary clinical features such as rod-cone dystrophy, obesity, renal dysfunction, reduced intelligence, polydactyly, male hypogonadism and secondary features such as hepatic fibrosis, diabetes mellitus, hypercholesterolaemia, reproductive abnormalities, short stature, speech defects and developmental delay. There is a progressive loss in visual acuity eventually leading to legal blindness in about 3/4 of the patients, usually by the second decade of life.

Genotype It is also genetically heterogeneous with 26 candidate genes identified so far: *BBS1*, *BBS2*, *ARL6* (BBS3), *BBS4*, *BBS5*, *MKKS* (BBS6), *BBS7*, *TTC8* (BBS8), *BBS9*, *BBS10*, *TRIM32* (BBS11), *BBS12*, *MKS1* (BBS13), *CEP290* (BBS14), *BBS15*, *SDCCAG8* (BBS16), *LZTFL1* (BBS17), *BBIP1* (BBS18), *IFT27* (BBS19), *BBS20*, *C8orf37* (BBS21), *ADIPOR1*, *IFT172*, *INPP5E*, *KCNJ13* and *NPHP1*. Oligogenic inheritance was also observed in BBS [60], and the presence of pathogenic mutations in different genes has resulted in severe disease phenotype in patients [61].

26.3.3 Senior–Loken Syndrome (SLSN)

Phenotype Senior-Loken syndrome is a renal-retinal syndrome which is a congenital, ciliopathic autosomal recessive disease that is characterised by nephronophthisis and Leber congenital amaurosis.

Genotype Genetically it is heterogeneous with seven genes and one locus associated with the disease. SLSN1 is caused by *NPHP1* (nephrocystin 1) gene, SLSN3 is associated with locus on chromosome 3q22, and SLSN4 is caused by mutation in *NPHP4* (nephroretinin) gene, SLSN5 by *NPHP5 (IQCB1)* gene, SLSN6 by *NPHP6 (CEP290)* gene, SLSN7 by *SDCCAG8* gene, SLSN8 by *WDR19* gene and SLSN9 by *TRAF3IP1* gene.

26.3.4 Refsum Syndrome (Batten Disease)

Refsum disease belongs to the peroxisome biogenesis disorders (PBD), which also include Zellweger syndrome, neonatal adrenoleukodystrophy and rhizomelic chondrodysplasia punctata.

Phenotype Refsum disease is an inborn error of lipid metabolism caused by deficiency of fatty acid alpha-oxidation. Clinically it is characterised by retinitis pigmentosa, anosmia, and cerebellar ataxia [62], and plasma phytanic acid levels are abnormal. Other systemic conditions include neuropathy, ichthyosis, cardiomyopathy, arrhythmia and eventually heart failure later in life.

Genotype RS is an autosomal recessive disease. Genetically, two genes were associated with the diseases – phytanoyl-CoA 2-hydroxylase gene (*PHYH*) where more than 90% of patients were shown to have mutation in this gene and *PEX7* gene where 10% of patients have shown mutations in this gene [63]. *Three more genes were found to be associated with the disease*, *namely*, *PEX1*, *PEX26 and PXMP3.*

26.3.5 Joubert Syndrome

Phenotype Joubert syndrome is a ciliopathic, neurodevelopmental disorder with heterogeneous clinical manifestations. Classic findings include the ‘molar tooth sign’ which comprises of malformation of the cerebellum and the brain stem and hypotonia and developmental delay. Ophthalmic manifestations include retinal dystrophy, abnormal eye movements and difficulty in smooth pursuits and gaze and tracking. It clinically overlaps with Bardet-Biedl syndrome and Meckel-Gruber syndrome.

Genotype JS is an inherited autosomal recessive disorder. Mutations in 33 genes have shown to be associated with Joubert syndrome.

JBTS1 is caused by *INPP5E* gene, JBTS2 by mutation in the *TMEM216* gene, JBTS3 by *AHI1* gene, JBTS4 by *NPHP1* gene, JBTS5 by *CEP290* gene, JBTS6 by *TMEM67* gene, JBTS7 by mutation in the *RPGRIP1L*, JBTS8 by *ARL13B*, JBTS9 by mutation in *CC2D2A*, JBTS10 by *CXORF5*(OFD1) gene, JBTS11 by *TTC21B* gene, JBTS12 by mutation in the *KIF7* gene, JBTS13 by *TCTN1* gene, JBTS14 by *TMEM237* gene, JBTS15 by *CEP41* gene, JBTS16 by mutation in the *TMEM138* gene, JBTS17 by *C5ORF42* gene mutation, JBTS18 by mutation in the *TCTN3* gene, JBTS19 by *ZNF423* gene, JBTS20 by *TMEM231* gene, JBTS21 by mutation in the *CSPP1* gene, JBTS22 caused by mutation in the *PDE6D* gene, JBTS23 by *KIAA0586* gene, JBTS24 caused by mutation in the *TCTN2* gene, JBTS25 by *CEP104* gene, JBTS26 by mutation in the *KIAA0556* gene, JBTS27 by *B9D1* gene, JBTS28 by mutation in the *MKS1* gene, JBTS29 by *TMEM107* gene and JBTS30 by mutation in the *ARMC9* gene [64]. Others genes associated recently are C2CD3 [65], CEP120 [66] and POC1B [67]. Only JBTS10 is X-linked recessive [68], and the rest all are inherited in autosomal recessive pattern. Early genetic diagnosis also helps in clinical management in Joubert syndrome as few systemic complications have a late onset like JBTS1 and 3 are restricted to central nervous system (CNS) defects, while JBTS2 is shown to have clinical impact on the kidney, retina and liver apart from CNS abnormalities [69].

26.3.6 Alagille Syndrome

Phenotype Alagille syndrome is an autosomal dominant disease with reduced penetrance, involving liver, heart, skeletal, ocular and facial abnormalities. Ophthalmic manifestations include posterior embryotoxon, iris abnormalities, retinal hypopigmentation with RPE speckling [70], angulated retinopathy [71] and optic nerve head abnormalities in about a third of the individuals. The ocular findings do not have major significance. Systemic manifestations include malformation of bile ducts, pulmonary artery hypoplasia with cardiac abnormalities and a butterfly-like vertebral arch.

Genotype So far two genes belonging to NOTCH signalling pathway are shown to be associated with Alagille syndrome – *JAG1* [72] and *NOTCH2* [73]. The majority of cases can be explained by sequence alterations in *JAG1*. *JAG1* is a transmembrane protein that constitutes a ligand in the notch pathway.

Since the notch signalling is an important developmental pathway, relevant for several tissues, it is not surprising that ALGS1 includes a wide range of clinical manifestations (hepatic, cardiac, vascular, skeletal, ocular, facial, renal, pancreatic and neuronal) [74]. Interestingly, the majority of the *JAG1* mutations are nonsense mutations or small deletions leading to premature termination codons. Furthermore, some deletions affect the entire coding region of *JAG1*. This supports the hypothesis that a reduced gene dosage (haploinsufficiency) may be the basis of the disease.

26.3.7 Alstrom Syndrome

Phenotype Alstrom syndrome is a rare ciliopathic disease with characteristic features of type II diabetes mellitus, retinal degeneration (cone-rod dystrophy or Leber congenital amaurosis), obesity and sensorineural deafness [75]. Other systemic associations include dilated or restrictive cardiomyopathy, insulin resistance syndrome, liver involvement with cirrhosis and multiple organ failure. Despite its similarities to Bardet-Biedl syndrome, mental retardation, polydactyly and hypergonadism do not belong to the repertoire of features in Alstrom syndrome.

Genotype Until now, mutations in *ALMS1* gene, located on chromosome 2, have only been associated with this disease in an autosomal recessive pattern. *ALMS1* is localised in the centrosomes, and they play an important role in centriole structure and function [76]. Accumulation of intracellular vesicles in the inner segments and

mislocalisation of rhodopsin to the outer nuclear layer indicate that *ALMS1* may play a role in intracellular trafficking .

26.3.8 Neuronal Ceroid Lipofuscinosis (NCN)

Phenotype Neuronal ceroid lipofuscinosis is a neurodegenerative disease caused due to impairment in lysosomal storage. They are characterised by the accumulation of autofluorescent material in different cell types including neurons. Clinical manifestations comprise epileptic seizures, progressive psychomotor retardation, visual loss and premature death. By the age of onset and clinical features presented, these are classified as congenital, infantile, late infantile, juvenile, adult and Northern epilepsy (epilepsy with mental retardation) [77].

Genotype It is also genetically heterogeneous with eight genes identified so far. The congenital NCN form is caused by *CTSD* (CLN10) gene. Infantile NCL is caused by *PPT1* (CLN1) and *KCTD7* (CLN1) gene. Late infantile form is associated with PPT1 (CLN1), *TPP1* (CLN2), *CLN5*, *CLN6*, *MFSD8* (CLN7), *CLN8* and *CTSD* genes (CLN10). Juvenile form is associated with *PPT1* (CLN1), *TPP1* (CLN2), *CLN3*, *CLN9* and *ATP13A2* (CLN12) genes. The Northern epilepsy variant is associated with *CLN8*. Adult NCL also known as Kufs disease is due to mutation in *CTSD* (CLN10), *PPT1* (CLN1), *CLN3*, *CLN5*, *CLN6* [78], *CTSF* (CLN13) and *GRN* (CLN11) genes. The above forms are autosomal recessive, while the autosomal dominant adult NCL, also known as Parry disease, is caused by *DNAJC5 (*CLN4B) gene [79].The most prevalent NCLs are juvenile-onset CLN3 and late infantile-onset CLN2 diseases.

26.3.9 Primary Ciliary Dyskinesias (PCD)

Phenotype Primary ciliary dyskinesias are a ciliopathic disorder involving motile cilia. It is also called oto-sino-pulmonary disease as it shows characteristic chronic abnormalities of the ear, sinus and lower airways. Approximately half of the patients have situs inversus (mirror orientation of thoraco-abdominal organs). Also, male infertility is common, and complex congenital heart disease can occur. The combination of situs inversus totalis, chronic sinusitis and bronchiectasis is also known as the Kartagener triad.

Almost all men with PCD are infertile from sperm immotility. Additional symptoms or clinical manifestations may include hydrocephalus, retinitis pigmentosa, polycystic kidney disease, liver cysts and biliary atresia. All those features are rare in PCD.

Genotype Genetically it is reported as an autosomal recessive disease in most cases involving 28 genes and 2 loci. Ciliary dyskinesia 1(CILD1) is caused by recessive mutation in *DNAI1* gene, CILD2 by *DNAAF3* gene, CILD3 by *DNAH5* gene, CILD5 by *HYDIN* gene, CILD6 by *TXNDC3* gene, CILD7 by *DNAH11* gene, CILD9 by *DNAI2* gene, CILD10 by *KTU* gene, CILD11 by *RSPH4A* gene, CILD12 by *RSPH*9 gene, CILD13 by *DNAAF1* gene, CILD14 by *CCDC39* gene, CILD15 by *CCDC40* gene, CILD16 by*DNAL*1 gene, CILD17 by*CCDC103* gene, CILD18 by *HEATR2* gene, CILD19 by *LRRC6* gene, CILD20 by *CCDC114* gene, CILD21 by *DRC1* gene, CILD22 by *ZMYND10* gene, CILD23 *by ARMC4* gene, CILD24 by *RSPH1* gene, CILD25 by *DYX1C1* gene, CILD26 by *C21ORF59* gene, CILD27 by *CCDC65* gene, CILD28 by *SPAG1* gene, CILD29 by *CCNO* gene, CILD30 by*CCDC151* gene, CILD32 by *RSPH3* gene, CILD33 by *GAS8* gene, CILD34 by *DNAJB13* gene, CILD35 by *TTC25* gene and CILD37 by *DNAH1* gene [80]. Apart from these the ciliary biogenesis proteins of *MCIDAS* [81] and *DNAH8* [82] are also associated with ciliary dyskinesias. The loci identified so far are CILD4 on 15q13 and CILD8 on 15q24-q25.

Apart from these X-linked recessive PCD is also reported along with other conditions such as mutation in *RPGR* in patients with X-linked reti-

nitis pigmentosa [83] and PCD and mutation in *OFD1* (CILD36) in patients with X-linked mental detardation [84] and PCD. Choroideremia is also reported as one of the triad in chromosome Xq21 deletion syndrome. It is X-linked recessive and results in mental retardation and congenital deafness along with choroideremia due to deletion of Xq21region [85] that encompasses *CHM*, *RSK4* and *POU3F4* deafness gene.

26.4 Conclusion

The structural organisation described in this review leads the reader through non-syndromic and syndromic forms of (i) rod-dominated diseases, (ii) cone-dominated diseases, (iii) generalised retinal degenerations and (iv) vitreoretinal disorders, caused by mutations in more than 165 genes. Clinical variability and genetic heterogeneity have an important impact on genetic testing and counselling of affected families. As phenotypes do not always correlate with the respective genotypes, it is of utmost importance.

that clinicians, geneticists, counsellors, diagnostic laboratories and basic researchers understand the relationships between phenotypic manifestations and specific genes, as well as mutations and pathophysiologic mechanisms.

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CYP1B1 Gene Mutation in Primary Congenital Glaucoma

27

Rita S. Sitorus

Abstract

Primary congenital glaucoma (PCG) is the most common childhood glaucoma affecting children from birth to age 3 years and is a major cause of blindness in this young population.

Mutations in the CYP1B1 gene, associated with GLC3 locus, have been found to cause PCG in children worldwide and are the dominant genetic cause for pediatric glaucoma in the Middle East and Central Europe. Our study in a small number of Indonesian and European PCG families supports the previous studies that reported mutations of the *CYP1B1* gene being responsible for the PCG phenotype. A different pattern of *CYP1B1* disease-causing mutations and benign variants appears to exist in Indonesian patients when compared to patients from other ethnic backgrounds.

Other genes such as LTBP2 and PXDN gene have been reported recently by several studies as being associated with pediatric glaucoma.

The exact mechanisms of how these gene abnormalities actually cause primary congenital glaucoma remain unclear. However, with the promising research progress so far, it is hoped that we will understand more about the pathogenesis of this disease and eventually potential new molecular targets to treat this threatening blindness disease in children will be developed in the near future.

Keywords

CYP1B1 · Gene · Mutation · Children · Primary congenital glaucoma

27.1 Introduction

Primary congenital glaucoma (PCG) is a rare form of glaucoma and is usually inherited in an autosomal recessive mode with incomplete penetrance [1]. PCG is the most common childhood glaucoma and one of the most important causes of childhood blindness. In a separate school for the blind study in Indonesia, we reported pediatric glaucoma, mostly the primary congenital form, accounted for 8.2% of the treatable causes of blindness in children [2].

PCG is caused by unknown developmental defect(s) of the trabecular meshwork and anterior chamber angle. It manifests clinically during the neonatal or infantile period. The disease is characterized by high intraocular pressure (IOP), buphthalmos with corneal enlargement, and breaks in Descemet's membrane.[1] In Saudi Arabia, the incidence is estimated to be 1:2500, whereas in western countries, the incidence is estimated to be less than 1:30000 [3, 4].

R. S. Sitorus (✉)
Department of Ophthalmology, Faculty of Medicine Universitas Indonesia, Cipto Mangunkusumo Hospital, Jakarta, Indonesia

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27.2 Clinical Features

Primary congenital glaucoma often manifests in the first years of life, especially during neonatal or infantile period. The later the onset, the less severe the defect, hence often leading to better prognosis. Also, the later the onset, the less clinical manifestation develops. Typically, it presents as triad of epiphora, blepharospasm, and photophobia. During the first year of life, glaucoma is often suspected whenever changes in scleral and corneal architecture are found, such as buphthalmos with corneal enlargement (Fig. 27.1).

Diagnosis is usually made based upon clinical findings. A comprehensive ophthalmologic assessment is mandatory due to pending diagnosis, and treatment may cause irreversible end-stage eye disease and permanent visual loss.

27.3 Genetic Aspects of PCG

PCG is a genetically heterogeneous disorder in terms of disease-associated loci, penetrance, and expressivity. To date, GLC3 loci have been determined with four specific loci identified: GLC3A (MIM 231300) on chromosome 2p22-p21, GLC3B on chromosome 1p36.2-36.1, GLC3C on chromosome 14q24.3, and GLC3D on chromosome 14q24.2-24.3 [5]. The GLC3A locus colocalizes to the *CYP1B1* gene (MIM 601771) on chromosome 2p21.

CYP1B1 is a member of the cytochrome P450 superfamily enzyme, which is the largest known enzyme of the human cytochrome P450 pathway that is primarily expressed in the trabecular meshwork, iris, retina, and ciliary body. This enzyme contributes to the development of normal trabecular meshwork microarchitecture by metabolizing essential molecules that are perhaps used in a signaling pathway, possibly a steroid [6].

The human CYP1B1 gene consists of three exons of which the first is noncoding. The putative open reading frame starts in the second exon and is 1629 bp in length [7–9]. More than 150 mutations including missense, nonsense, regulatory, and insertions and/or deletions in *CYP1B1* have been associated with PCG [5] and are the main known cause of PCG. In ethnically mixed populations, mutations were found in 20–30% of patients with PCG,[11] whereas in consanguineous populations, the prevalence increases to 85% [3, 8, 10].

27.4 Genetic Analysis of *CYP1B1* in Indonesian and European Patients

We screened the coding region of the CYP1B1 gene in 21 patients clinically diagnosed as congenital glaucoma [11]. Twelve of them were of Indonesian descent and nine of European descent. Blood samples were obtained from subjects and their relatives. We use oligonucleotides primers reported by Bejjani et al. to amplify the coding exons of the CYP1B1 gene by performing PCR analysis [3]. Subsequently, PCR products were analyzed using single-strand conformation polymorphism analysis (SSCP).

Our study identified *CYP1B1* mutations in 33.3% and 22.2% of subjects screened from Indonesian and European patients, respectively [11]. Five distinct disease-causing mutations were identified in 6 of the 21 index cases: V364 M, S215I, E281X, 1407del12bp, and 1410del13bp.

In the Indonesian patients, the missense mutations V364 M (3/12 patients) and S215I (1/12 patients) were identified in Indonesian patients. Mutations were found exclusively in Indonesian-Sundanese patients, which suggest that V364 M is the most common mutation in this ethnic group. We ruled out V364 M as recent founder mutation in this ethnic group by testing six short sequence repeat markers at 2p24–12. Alleles varied among all tested patients for the informative

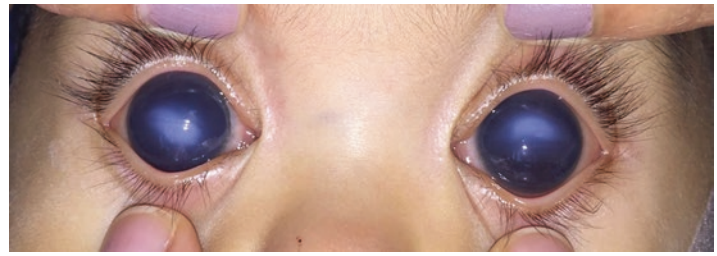

Fig. 27.1 A 9-month infant with Primary Congenital Glaucoma, showing buphthalmos, corneal haze, and increased intraocular pressure

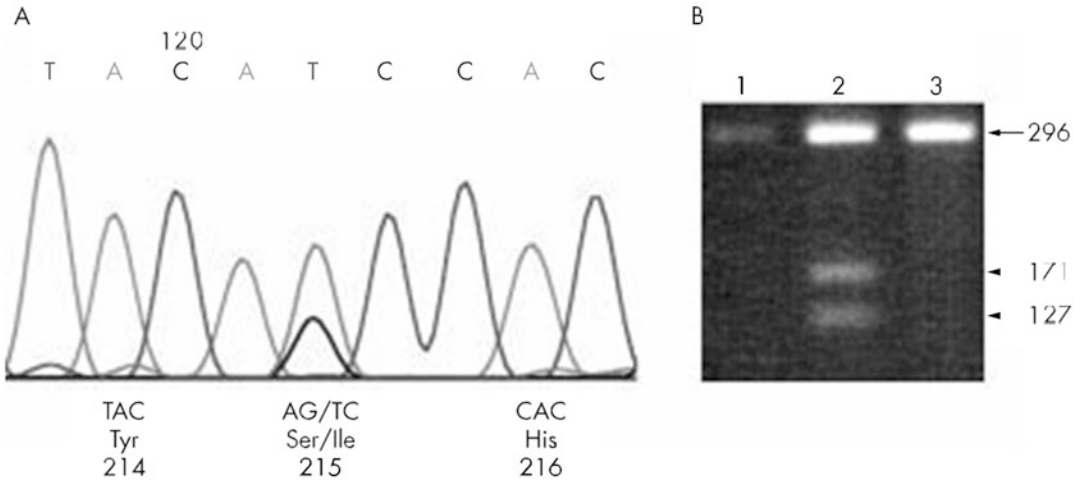

Fig. 27.2 Direct sequencing of the 5′ amplimer of exon 2 in case 7. (**a**) Direct sequencing representing a novel mutation S215I . A G → T transition at the second nucleotide in codon 215 in exon 2 leads to the substitution of a Ile residue for a Ser residue. (**b**) FokI restriction endonuclease digest (lanes 1 and 3, controls; lane 2, case 7)

markers. The V364 M mutation has been reported in a Japanese study in the compound heterozygous state in 1 of 11 families [17]. This mutation, however, has never been reported in white populations, and we also did not find this mutation in our European patients. The possibility exists that the mutation originated from an ancient founder and then spread throughout Asia, since even cases 1 and 2 (sibs) shared only one allele for some markers, despite being the product of a consanguineous marriage. The V364 M mutation might also have occurred independently owing to a mutational hot spot caused by the surrounding sequence (GTC *G*TG GGG).

S215I mutation, a serine to isoleucine transition affecting the second nucleotide of codon 215 in exon 2, was identified in an Indonesian patient and is a novel mutation (Fig. 27.2). It was singly heterozygous (only one mutant allele was detected). No genomic DNA from the unaffected parents was provided for segregation analysis; however, this missense mutation was not present in 50 chromosomes from randomly selected normal subjects.

In a Saudi Arabian PCG study, other distinct missense mutations G61E, R468W, and D374N were reported as the most common mutations, accounting for 72%, 12%, and 7% of the tested alleles, respectively. In Brazilian patients, a single truncating mutation g.4340delG (20.2%) was the most frequent mutation and was different from Turkish PCG families who showed an equal frequency of truncating and missense mutations in the tested samples [6, 12].

In contrast to the Indonesian PCG families, the truncating mutations account for the majority of the *CYP1B1* mutations identified in the European PCG families (Table 27.1). All of the mutations identified probably truncate the open reading frame. They include one nonsense mutation (E281X), one frameshift mutation (c.1410del13bp), and one in-frame mutation (c.1407del12bp). These mutations are expected to eliminate amino acids 79–377 from the carboxy-terminus of the *CYP1B1* polypeptide. Thus, if a stable protein is synthesized, every mutant molecule would lack at least the heme-binding region, which is essential for the function of the cytochrome P450 molecule. Therefore, it is expected that these mutations will result in functional null alleles.

Peters' anomaly has been reported as being correlated with mutation in *CYP1B1* [13, 14]. In our study, we identified E281X/del355RVGA mutation (case 5). It appears that this entity does not result from a single specific mutation or type of mutation since in our case, the combination of a stop mutation and a deletion shows a similar phenotype to a stop mutation (W57X) and a missense change (M1T), which by themselves do not predict the phenotype of Peters' anomaly.

Different pattern of single nucleotide polymorphisms (SNPs) appears to exist in the Indonesian population compared to European (white) patients. Benign sequence variants such as A453S and L432 V have been identified in European patients only, while the allele frequency of the noncoding variant IVS1-12 t/c was much higher in European than in Indonesian patients (60% compared to 15.8%).

However, as in other reported studies [12, 16], we also found that R48G and A119S were always co-inherited, indicating that these two SNPs are linked [16]. We could not confirm the hypothesis that the combination of four well-known polymorphisms, R48G, A119S, L432 V, and N453S, establishes a pathogenic allele when co-inherited, as previously reported [17].

Many studies have demonstrated *CYP1B1* as a critical gene responsible for PCG [3–6, 8, 10–12, 15, 17, 19–28], which is directly related to the integrity of trabecular meshwork. CYP1B1 participates in the normal development and function of the eye by metabolizing essential molecules that are probably used in a signaling pathway [6]. The detailed mechanism of *CYP1B1* alteration which may contribute to the pathogenesis of PCG has, however, remained unclear [28].

27.5 *CYP1B1* Mutation Reported in Various Population with PCG

Various disease-causing mutations in CY1B1 gene recently reported from different populations were shown in Table 27.2.

CYP1B1 mutations occur in 87% of familial and 27% of sporadic cases of PCG worldwide [6]. Five major groups of mutations in *CYP1B1* have been found in PCG: missense mutations, frameshift or truncating mutations, mutations triggering the nonsense-mediated mRNA decay of C*YP1B1* [6, 28], and mutations in the promoter or control regions of the gene.

27.6 Other Genes Associated with PCG

Several studies reported LTBP2 and PXDN genes to be associated with pediatric glaucoma. A new locus (*GLC3D*) harboring the *LTBP2* gene has been characterized in developmental glaucoma, but its role in classical cases of PCG is yet to be understood.

Ali M et al. [20] reported null mutations in LTBP2 cause PCG in four consanguineous families from Pakistan and in patients of Gypsy ethnicity. LTBP2 maps to chromosome 14q24.3 but is around 1.3 Mb proximal to the documented GLC3C locus. It remains to be determined

Table 27.1 CYP1B1 gene: genotype-phenotype correlations on mutations reported in our study [11]

Case	Gender	Sequence change allele1/allele2	Predicted effect	Exon	Ethnic background	Consanguinity	Age at diagnosis	Age at onset	Therapy, started at age	IOP at diagnosis	Visual acuity RE/LE
1	F	c.1436G-A homozygous	V364 M homozygous	3	Indonesian-Sundanese	Yes	19 y	<3 y	M, 19 y	High/30–40	HM/P*
2	M	c.1436G-A homozygous	V364 M homozygous	3	Indonesian-Sundanese	Yes	15 y	<3 y	M, 15 y	High/30–40	LP/LP*
3	M	c.1436G-A homozygous	V364 M homozygous	3	Indonesian-Sundanese	Yes	15 y	<3 y	M, 15 y	High/30–40	LP/LP*
4	M	c.1436G-A/ND	V364 M homozygous	3	Indonesian-Sundanese	No	16 y	<3 y	M, 16 y	High/30–40	LP/LP*
5	F	c.1189G-T/1407del12bp	E281X/ del355RVGA	2/3	Turkish	No	3 mth	Congenital	TET, 3 mth CPC, 11 mth CPC, 3 y	25/25	1,0/1,0
6	F	c.1410del13bp/ND	Frameshift/ND	3	Italian	No	2 mth	4 d	T, 9 mth	17/20	1,6/2,4 cyc/degree
7	M	c.992G-T/ND	S215I/ND	2	Indonesian-Sundanese	No	24 y	<3 y	M, 24 y	High/30–40	LP/LP*

**HM* hand movement, *LP* light perception. †By PL + TAC procedure; M, pilocarpine + acetazolamide, CPC: sequence data refer to GenBank entry HSU56438 and the sequence given by Sutter et al. [18] and Stoilov et al. [19]

Table 27.2 *CYP1B1* disease-causing mutation recently reported in patients with PCG in various population

No.	Mutations	Population	References
1	p.F231I, p.P437A, p.G61E, c.535delG	Tunisia	Bouyacoub [20]
2	p.Arg355*/p.Arg355, p.Arg390Cys/c.1209_1210insTCATGCCACC, p.Glu387Lys/c.1209_1210insTCATGCCACC, p.Trp57*/c.1209_1210insTCATGCCACC, p.Arg355*/c.1209_1210insTCATGCCACC, p.Glu387Lys/c.1064_1076delGAGTGCAGGCAGA, p.Trp57*/p.Ala106Asp	United States	Lim [21]
3	p.Arg355fsX69, p.Thr404fsX30, p.Arg469Trp, p.Thr404fsX30	Spanish	Milla [22]
	162-kb del(2p21.1), p.Asp449fsX6, p.Arg368His, p.Trp57Stop, p.Ser464PhefsX12, p.Gly61Glu, p.Leu277Stop		
4	c.970_971dupAT; p.T325SfsX104, p.G329S, p.V419Gfs11X	Korean	Kim [23]
5	g.4339delG (predominant), p.R163C, p.C470Y, g.4330-4331delTG, p.R163C, p.E173K, g.4330-4331delTG, p.E229K, p.R390S, p.R368H, p.R469W, p.C470Y, g.7901-7913del13bp	Moroccan	Hilal [24]
6	A106D, E173X, F261 L, E262X, W341X, P513_K514del	Spanish	Campos-Mollo [25]
	P52L, G61E, Y81N, E229K, P400S		
7	g.3972delC, g.4168_4169insGACCGGCCGGCCTTCGCC, g.8209_8213delAGCAGinsTTGTTGAAAAA, p.I60M, p.V95A, p.L107 V, p.P118S, p.F134S, p.N203S, p.A287S, p.D291G, p.N319S, p.V363D, p.R368L	Chinese	Chen [26]
8	E173K. N498D, G61E	Egyptian and Saudi Arabian	El-Ashry [27]

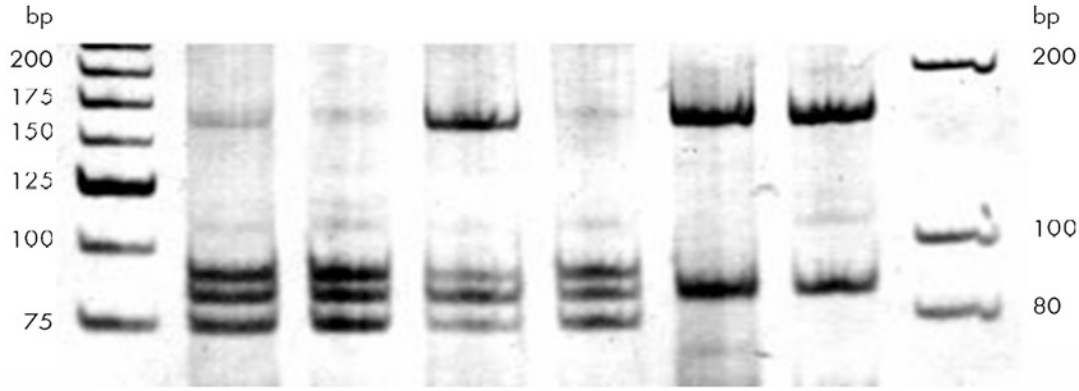

Fig. 27.3 Mutation detection and confirmation of the V364 M mutation. NlaIII restriction endonuclease digest. Lane 1, case 1; lane 2, case 2; lane 3, case 4; lane 4, case 3; lanes 5 and 6, controls; lane M1, marker 25 bp ladder; lane M2, marker 100 bp ladder

Direct sequencing of the wild-type amplimer indicating the amino acids Arg 355, Val 356, Gln 357, and Ala 358 abolished by the deletion

whether LTBP2 is the GLC3C gene or whether a second adjacent gene is also implicated in PCG.

LTBP2 mutations were also identified in autosomal recessive congenital/infantile glaucoma with the clinical spectrum of primary megalocornea, spherophakia with ectopic lens, and lens-related glaucoma; however the glaucoma may be secondary (lens-related) rather than primary. LTBP2 DNA sequencing of individuals with primary congenital glaucoma identified 14 sequence variants comprising 7 transitions and 7 transversions. These variants were inherited in a hetero-

zygous manner and included 3 coding nonsynonymous and 11 coding synonymous SNVs [21]. PXDN gene is also reported to cause developmental glaucoma with opacification of the cornea and cataract [29].

27.7 Conclusion

CYP1B1 has been approved by many studies as an important gene responsible in PCG, which is directly related to trabecular meshwork integrity.

Our study supports the previous findings that mutations in the *CYP1B1* gene are responsible for the PCG phenotype that is associated with the GLC3A locus. A different pattern of *CYP1B1* disease-causing mutations and benign variants appears to exist in Indonesian patients when compared to patients from other ethnic backgrounds. V364 M mutation has been shown as the most common mutation in our screening analysis.

The clear mechanisms by which CYP1B1 plays a role in the pathogenesis of this disease are still under investigation. With the promising research progress, a better understanding of the pathogenesis of this disease at the molecular level will eventually lead us to the hope of developing new molecular targets for PCG treatment in the future.

Acknowledgments Table 27.1, Figs. 27.2 and 27.3, and some quotes [11] were reproduced from "CYP1B1 gene analysis in primary congenital glaucoma in Indonesian and European patients." Sitorus R, Ardjo SM, Lorenz B, Preising M, Journal of Medical Genetics 2003:40;e9, with permission from BMJ Publishing Group Ltd.

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28 Diabetic Retinopathy: Clinical, Genetic, and Health Economics (An Asian Perspective)

Siddhita Nare, Sunita Mohan, Uthra Satagopan, Sundaram Natarajan, and Govindasamy Kumaramanickavel

Abstract

Diabetes mellitus is the fastest growing disease in the world that is estimated to reach nearly half a billion in 2045, and a third of them would have microvascular complication like diabetic retinopathy (DR). Hyperglycemia, hypertension, and dyslipidemia are some of the controllable risk factors. DR is classified into nonproliferative, proliferative, and macular edema types. Many molecular factors like *VEGF*, *ALR2*, *eNOS*, *MTHFR*, *ACE*, *IGF*, and *RAGE* and its associated single nucleotide polymorphisms play a critical role in the process of neovascularization. Some of the drug discovery and newer treatment regimens are based on these molecular factors. More research by the clinicians, epidemiologists, and vision scientists is necessary to reduce the visual morbidity and disease burden of DR in the community.

Keywords

Diabetic retinopathy · Health economics · Genetic susceptibility · Type 2 diabetes mellitus · Prevalence

S. Nare (✉) · S. Mohan (✉) · S. Natarajan
G. Kumaramanickavel
Aditya Jyot Foundation for Twinkling Little Eyes, Mumbai, Maharashtra, India

U. Satagopan
Centre for Medical Genetics, Chennai, India

28.1 Introduction

28.1.1 Prevalence of Diabetes Mellitus (DM) and Diabetic Retinopathy (DR)

Diabetes mellitus (DM), a noncommunicable complexly origin disease, is considered as one of the most challenging health problems rising at a tremendous pace globally. DM is estimated to rise to 629 million by 2045 from 425 million in 2017 [1]. According to IDF 2017 report, 159 million in Western Pacific and 82 million in Southeast Asian adults are living with DM [1]. China, India, Indonesia, and Pakistan represent 48% of the global burden [1]. With the incidence of DM increasing at an alarming rate, the number of people with diabetic retinopathy (DR), a microvascular complication, is expected to surge from 126.6 million in 2010 to 191.0 million by 2030 [2]. DR ranks the fifth most common cause of global blindness (moderate and severe vision impairment) [3] accounting for 4.8% of the cases of blindness throughout the world [4]. It is one of the leading causes of visual impairment and blindness in the working-age population (24–70 years) in both developing and developed countries [5].

A pooled analysis of 22,896 people with DM from 35 population-based studies in the USA, Australia, Europe, and Asia (between 1980 and 2008) showed that the overall prevalence of

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any DR in T1DM (type 1 diabetes mellitus) and T2DM (type 2 diabetes mellitus)) was 34.6% (95%CI 34.5–34.8), with 10.2% having vision-threatening diabetic retinopathy (VTDR) [6].

The prevalence of DR appears to be higher in patients with T1DM than in those with T2DM [3]. Nearly all persons suffering from T1DM develop retinopathy, while more than 77% of persons with T2DM may develop retinopathy after 20 years' duration of diabetes [5], and approximately 25% of persons with DR may develop macular edema [7]. In India, the overall crude prevalence of DM is 4.7% in urban and 1.9% in rural areas [8], and 7.3–26.2% of these develop DR [9]. In a population-based door-to-door survey in the urban slums of Western India, we reported the DR prevalence of 1.41% in general population and 15.4% in type 2 DM [10]. The prevalence of severe retinopathy in patients with T1DM has diminished over the past 35 years due to improved medical care [11], but the recent epidemic of T2DM requires a new understanding of the biology of DM. The growing number of diabetic patients and the longer life span in aging population imply an increase in patients suffering from DR, which not only affects the quality of life of the individuals and their families but also increases the medical and economic burden of the society.

28.1.2 Social Burden and Health Economics

DM and its complications like DR impose a huge economic burden on the global healthcare. According to the International Diabetes Federation (IDF) estimation, globally USD 727 billion is spent for DM (20–79 years age group), whereas USD 850 billion was spent (19–99 years age group) in 2017. The expenditure on diabetes is projected to reach USD 776 billion (20–79 years age group) and USD 958 billion (18–99 years age group) by 2045 [1]. A recently published study from India estimated that the total annual expenditure on DM care was, on an average, INR (Indian rupee) 10,000 (USD 154) in urban areas and INR 6260 (USD 96.42) in rural areas [12]. Patients with DM having retinal complication spent approximately INR 13922 (USD 214.38) per month [12]. For a chronic disease like DM and hence DR, there is an unmet urgency for development of a cost-effective therapy that is dependent on a basic understanding of the pathophysiological progression of DR.

28.2 Patho-mechanisms and Biology of Retinopathy

Retinopathy is a slow-progressing disease, mainly characterized by damage of the microvasculature of the retina. The onset and progression of retinopathy are triggered by numerous factors including extended duration of diabetes, poor control of blood glucose, elevated blood pressure, and dyslipidemia [13–16].

Histological studies, using postmortem retinas of diabetic patients, have revealed several cellular changes: selective endothelial and mural cell loss (including pericytes), presence of mural cell ghosts, endothelial clusters, acellularity and microaneurysms [17, 18], basement membrane thickening, presence of hemorrhage in the inner nuclear layer (INL) and outer plexiform layer (OPL), as well as eosinophilic exudates in the OPL [18] .

Further, immunological and immunohistochemical studies have shown hypertrophy of Müller cells throughout the inner and outer diabetic retina and increased apoptosis [19]; expression of pro- and antiapoptotic molecules in ganglion and glial cells, respectively [20]; and elevated levels of vascular endothelial growth factor (VEGF) in retinal blood vessels of diabetic patients with pre-proliferative or no retinopathy stages [21]. Alternation in several other factors, including somatostatin [22], cortistatin [23], αA- and αB-crystallins, advanced glycation end products (AGEs), and receptor for AGE (RAGE) [24], as well as apolipoprotein A1 (ApoA1) [25], was also observed in the postmortem tissues.

Advanced molecular studies revealed abnormal levels of expression of mRNA and proteins of various chemokines [26, 27], cytokines [26–29], inflammatory markers [29–31], and

angiogenic factors [27, 29, 30, 32] in aqueous humor, serum, or urine from diabetic patients. Although these morphological and molecular studies provide a better picture of the pathogenesis of DR at a cell and molecular level, they failed to provide mechanistic biological pathway. However, based on these observations, several mechanisms and interlinked biological pathways such as hyperglycemia and oxidative stress-mediated AGE products and inflammation, endoplasmic stress (ES)-mediated unfolded protein response (UPR) and apoptosis, hypoxia- and ischemia-mediated angiogenesis, activation of protein kinase C, polyol production, and hexosamine pathways have been postulated to be responsible for retinopathy complications in DM [33–36]. As these mechanisms and pathways are interlinked [37], the strategies to prevent the development/progression of this complication become complicated.

28.3 Clinical Diagnosis and Classification of DR

From a clinical standpoint, it is clear that the primary driving factors in DR pathogenesis are uncontrolled hyperglycemia, hypertension, and dyslipidemia. However, recent evidences have also suggested neurodegeneration as an early event in the pathogenesis of DR [38].

The diagnosis of DR essentially remains clinical in nature, the gold standard being dilated eye exam and serial fundus images. DR is classified as either nonproliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR) based on the presence of neovascularization that typifies the proliferative form [13]. Nonproliferative features of retinopathy, include microaneurysms, intraretinal hemorrhage, hard exudates, venous beading, and intraretinal microvascular anomalies (IRMAs), and the proliferative form features the neovascularization (NV) that bleeds easily resulting in vitreous hemorrhage, subsequent fibrosis, and tractional retinal detachment [39, 40]. DME characterized by increased vascular permeability and deposition of hard exudate at the central retina secondary to lipoproteins leaking from retinal capillaries into the extracellular space of the retina [41] is also a major cause of vision loss and can occur at any stage of DR.

IDF estimates globally 64% of people are living with DME, while 58% with DR face difficulties in performing daily activities [1]. Vision loss results from retinal detachment if patients are left untreated. Several emerging automated technologies have demonstrated promise in assisting with the diagnosis of sight-threatening DR leading to prompt referral for the timely management of the treatable conditions.

28.4 Genetics and Epigenetics of DR

DR is a complex disease, strongly influenced by both genetics and environment. Single nucleotide polymorphisms in genes encoding for the molecules and other enzymes, cytokines, and growth factors involved in DR pathophysiology have been associated with risk for DR in various populations. Among the candidate genes studied for variations and association with DR, *VEGF*, aldose reductase (*ALR2*), endothelial nitric oxide synthase (*eNOS*), methylene tetrahydrofolate reductase (*MTHFR*), and *RAGE* have been widely studied in various populations (Table 28.1).

Elevated serum and vitreous VEGF levels have been associated with PDR suggesting possible increase in *VEGF* gene expression in DR. The C(−634)G promoter *VEGF* polymorphism has been associated with risk for DR [67] and DME in Japanese cohorts [68], similarly G(−1154)A and C(− 7)T, and T(−1498)C polymorphisms are reported to be associated with risk for DR in Caucasian [69] and South Indian populations [70], respectively. *VEGF*-460C variation might accelerate the pathogenesis of retinal neovascularization in T2DM patients as suggested by association studies in Indian population [59]. In Chinese patients with T2DM, SNPs rs699947, rs833061, and rs13207351 at the promoter region of the *VEGF* gene might have association with predisposition DR [71].

Table 28.1 Genetic association studies for DR, PDR, and DME in various population

Gene	Chromosome location	Variation	Disease	Type	Association	Population	References
AR	7q35	C [-106] T, CC genotype	DR	T2DM	Significant association—risk	Iranian, Japanese, Egyptian	[42, 43]
			DR	T2DM	No significant association	Chinese	[44]
		C [-106] T, C allele	DR	T1DM	Significant association—risk	Asia, South America, Europe, and Australia	[45]
AKR1B1		-106C>T, [rs759853], TT genotype	DR	T2DM	Significant association—risk	North Indian population	[46]
		Z-2 Microsatellite	DR	T2DM	High risk	Asian Indian	[47]
			DR	T1DM/T2DM	Confer risk	White Ancestry	[48]
			DR	T2DM	Involved in the development of DR	Japanese population	[49]
			DR	T2DM	early onset of DR	Chinese population in Hong Kong	[50]
			DR	T2DM	Risk	Caucasian	[51]
		C[-12]G	DR	T2DM	Risk	China	[52]
		Z-4	DR	T1DM	Risk	Japanese	[53]
		Z+2	DR	T1DM	Confer protection against DR	White Ancestry	[48]
ALAR2		Z+2	DR	T2DM	associated with susceptibility to DR	South Indian Cohort	[54]
eNOS	7q36	VNTR 4b/a, a allele	PDR	T2DM	Significant association—risk	Slovenian	[45]
		T-786C & C774T	DR	T1DM	Risk for early onset severe DR	French	[55]
		Intron4 VNTR	DR	T2DM	Risk	Chinese, German, Japanese	[56]
			DME	T2DM	Risk	Japanese	[56]
RAGE	6p21.3	Gly82Ser, Ser82genotype	DR	T2DM	Significant association—risk	North Indian, Chinese	[45]
		-374A T/A	Sight threatening DR	T1DM	Risk associated	Scandinavian origin	[57]
		1704T allele	DR	DM	Risk associated	East Asian	[58]

VEGF	6p21	[-460 C/T], C allele	PDR, DR	T2DM	Significant association—risk	Indian, Caucasian	[45, 59]
		[+936C/T]	DR	T2DM	Positive association—risk	Asian	[45]
		[-2578C/A]	DR	T2DM	Significant association—risk	Chinese	[45]
		rs13207351	DR	T2DM	Positive association—risk	Chinese	[45]
		rs699947[-2578 A/C]	DR	T2DM	Positive association—risk	Asians and Europeans	[45]
		rs699947[-2578C/A]	DR	T2DM	Positive association—risk	Asians and Caucasians	[45]
		rs3025039[+936 C/T]	PDR	T2DM	Positive association—risk	Bengali Hindu	[45]
		rs2010963 [+405 G/C]	PDR	T2DM	Positive association—risk	Bengali Hindu	[45]
		rs2010963[-634G/C], C allele	DR	T2DM	Positive association—risk	Caucasian, Japanese	[45]
		rs2010963[-634G/C], C allele	DME	T2DM	Risk	Japanese	[45]
		+405CC genotype	DR	T2DM	Risk associated	Japanese	[60]
		CG genotype	DR	T2DM	Risk associated	Indian	[60]
		[-1154]C	DR	T2DM	Risk	Caucasian	[56]
		C[-634]G	DR	T2DM	Increase the risk for DR in patients with microalbuminuria	South Indian Cohort	[61]
ACE	17q23	Deletion polymorphism	Advanced DR	T2DM	Risk	Japanese	[56]
		Insertion/ Deletion	DR/NPDR	T2DM	Risk	Pakistani	[62]
PPARγ	3p25	Gly482Ser	DR	T2DM	Risk	Slovenia	[56]
Vit D receptor	12q13	Fok1 polymorphism	DR	T1DM	Risk	French	[56]
		Taq 1 Polymorphism	DR	T1DM	Tt- Risk in poor glycemic	French	[56]
Adiponectin	3q27	G276T	DR	T2DM	Risk	Japanese	[56]
MTHFR	1q36	C677T	DR	T2DM	Risk	Chinese	[56]
Glycoprotein	5q23	807T	Advanced stages of DR	T2DM	Risk	Sweden	[56]
TGFβ	9q13	R25P	PDR	T2DM	Risk	Caucasian	[56]
Neuro-peptide Y	7p15	L7P	DR	T2DM	Risk	Finnish	[56]
Alpha2/beta1 integrin	17q21	Intron 7 polymorphism	DR	T2DM	Risk	Japanese	[56]
HLA	6p21	DR9 & DQA1	DR	T1DM	Risk	Senegal	[56]

(continued)

Table 28.1 (continued)

Gene	Chromosome location	Variation	Disease	Type	Association	Population	References
MCP-I	17q11.2	rs1024611 [-2518 A/G] AA genotype	PDR	T2DM	Positive association—risk	Korean	[45]
		rs1024611 [-2518 A/G] G allele	PDR & NPDR	T2DM	Positive association—risk	Han Chinese	[45]
		rs1024611 [-2518 A/G] G allele	DR	T2DM	Increased onset	Japanese	[45]
MnSOD	6q25.3	A16V[C47T] AV genotype	DR	DM	Positive association—risk	North Iranian	[45]
iNOS	17q11.2	13-repeat genotype	DR	T2DM	associated with susceptibility to DR	South Indian Cohort	[54]
TNF	6p21.3	15-repeat genotype [β gene]	DR	T2DM	associated with susceptibility to DR	South Indian Cohort	[54]
		NcoI	PDR	T2DM	β2 allele is genetic factor for incidence of PDR	Caucasian – Slovak	[63]
		[GT]n microsatellite	DR, PDR	T2DM	Allele 4 [103 bp] is a low risk for developing retinopathy, Allele 8 [111 bp] is associated with PDR	Asian Indian	[64]
PEDF gene polymorphism	17p13.1	T130T	DR	T2DM	Moderate protective association	South Indian Cohort	[65]
IGF-1	12q23.2	promoter [CA] 18 repeat genotype	DR	T2DM for more than 15 years	high risk for developing DR and PDR	Southern Indian sample cohort	[66]

Among Chinese Han individuals with T2DM, polymorphism -634G/C of the *VEGF* gene was not correlated with NPDR or PDR; however, polymorphism-460C/T of the *VEGF* gene was correlated with NPDR, and C allele was associated with lower NPDR risk than T allele [72].

The gene *ALR2* that codes for aldose reductase, the rate-limiting enzyme of the polyol pathway, has a particular Z-2 promoter microsatellite repeat which has not only been associated with genetic susceptibility to DR in Caucasian [48, 73] and Asian Indian populations [47] with T2DM but also has been shown through functional studies to enhance gene expression in response to hyperglycemia. Also an association was observed between DR and the C-106 T, CC genotype in the T2DM patients in Iranian [42] and Japanese population [74] but not in Chinese population [44]. Kaur et al. reported significant association of *AKR1B1* -106C > T polymorphism (homozygous recessive TT genotype) with retinopathy in North Indian patients [75]. Studies from Asia, South America, Europe, and Australia showed an association between C(−106)T polymorphism and the risk of DR in T1DM but not type 2 DM [45]. The Z-4 allele was significantly associated with patients with proliferative retinopathy in Japanese population. While the same study reported an association of Z + 2 allele with patients without retinopathy [53], a South Indian study reported an association of Z + 2 allele with risk for DR [54]. Results on Z + 2 allele from T1DM white ancestors were comparable to Japanese study [48].

Increased production of nitric oxide by down-regulation of *eNOS* has been shown to result in angiogenesis in animal models [76]. The intron4 27-bp (*VNTR)* has been consistently associated with risk for DR in Japanese [77], German, and Caucasian [78] populations.

Ser82 allele in the *RAGE* gene is a low-risk allele for developing DR in Asian Indian patients with T2DM [54, 79], whereas Vanita V. showed significant association of p.Gly82Ser polymorphism in *RAGE* with DR in T2DM patients [80]. However, studies from Malaysia, the USA, Europe, and Asia reported no associations between *RAGE* polymorphisms and DR [45]. Lindholam (2006) reported an association between *RAGE*(−374 T/A) polymorphism and type 1 diabetes [57].

Saleem et al. (2015) observed a significant association between insertion deletion polymorphism rs4646994 in intron 16 and DR and NPDR, but not with PDR in Pakistani cohort [62]. Matsumoto reported significant association in Japanese population between the presence of the D allele polymorphism in the *ACE* gene and advanced diabetic retinopathy (ADR) in Japanese subjects with T2DM [81].

An 18-repeat polymorphism in the promoter of IGF-1 gene is a susceptibility genotype for DR, and its clinical severity in a Southern Indian cohort is found by Uthra et al. [66] who also reported lack of association of PRKCB1 gene promoter polymorphisms and moderate protective association of PEDF gene polymorphism with DR in the same cohort [65].

Monocyte chemoattractant protein-1 (*MCP-1*) is a chemokine specific for monocytes and basophils. *MCP-1* rs1024611 (−2518 A/G) AA genotype was significantly associated with PDR in T2DM in Korean patients. On the other hand, the G allele of the same polymorphism in Han Chinese patients was significantly associated with high-risk PDR in T2DM. A study in Japanese patients with T2DM also reported that the G allele was significantly associated with DR [45].

Single gene association studies have not been comprehensively informative about the role of the DNA polymorphisms in disease pathogenesis, and hence approaches like haplotype analysis, linkage disequilibrium, and functional studies are expected to throw more light in this area.

Analysis of a handful of genetic variations is often insufficient to understand the genetic etiopathology of polygenic disease as DR. Hence, researchers now rely on robust technologies such as genome-wide association studies. Imperatore et al. (1998), in their study on Pima Indians with T2DM, showed some evidence of linkage to chromosomes 3 (LOD = 1.36) and 9 (LOD = 1.46) for diabetic retinopathy, although the evidence was insufficient for genome-aide studies [82]. Looker et al. (2007) performed a genome-linkage

analysis for DR and found evidence of linkage to chromosome 1p (LOD = 3.1 by single-point analysis and 2.58 by multipoint analysis) [83]. Similarly, another study on Mexican Americans with T2DM revealed suggestive linkage on chromosome 3 (LOD = 3.41) and chromosome 12 (LOD = 2.47) [84]. However, fine mapping of critical genomic regions, which harbor possible susceptibility genes, has not yet been reported. Genome-wide meta-analysis performed by Grassi et al. (2011) identified an intragenic SNP on chromosome 6 rs227455, located more than 200 kb from two undesignated genes *LOC728275* and *LOC728316*, and rs10521145 in strong linkage disequilibrium with copy number variation CNVR6685.1 on chromosome 16 to have strong association with risk for sight-threatening DR [85]. A genome-wide association study on Taiwanese population reported a risk association of SNPs located in five novel chromosomal regions in and around *MYSM1*, *PLXDC2*, *HS6ST3*, and *ARHGAP22* genes; the latter two found to have significant role in endothelial cell angiogenesis and increased capillary permeability [86]. A three-stage genome-wide association study carried out in a Japanese cohort revealed a borderline significance of association of an intronic SNP in long intragenic noncoding RNA RP1-90 L14 adjacent to *KIAA1009/QN1/CEP162* gene, suggesting a possible role of ciliary-associated genes in the pathogenesis of DR due to the involvement of *CEP162* gene [87].

However, identification of genes and genetic variations conferring risk for the development of DR and recognition of pre-symptomatic individuals would have tremendous impact in the treatment of the disease-related complications and could be useful in genetic counselling.

28.5 Recent Advances in Genetics/Epigenetics in Understanding and Management of DR

With the current use of inhibitors for growth factors involved in the DR pathology such as bevacizumab, ranibizumab, and aflibercept (VEGF-Trap) [88], significant genotype-specific personalized management strategies for patients could evolve. Gene therapy experiments targeting renin angiotensin system (RAS) pathway and antioxidant enzyme activities are also underway owing to the limitations of the current inhibitor-based treatments, which collectively hold promise in the effective management of diabetic microvascular complications in the retina [89].

28.6 Future Trends

Research in DR has to be a concerted effort between the ophthalmologist, the epidemiologist, and the vision scientist. Such efforts have led to the current knowledge that we have in the field.

However, further research is needed to identify molecular genetics and biological factors that could be applied as early genetic or biomarkers to identify the target population to reduce the burden of visual impairment or blindness in a community. Besides, such pathway analysis would also lead to the discovery of newer drugs that would reduce the morbidity or disease burden. Granting agencies should focus on these areas of research as DM is one of the rapidly rising diseases with epidemic proportions.

Conflict of Interest None of the authors have any proprietary interests or conflicts of interest related to this submission.

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Glaucoma Genes in East Asian Studies

29

Shi Yao Lu, Clement C. Y. Tham, Pancy O. S. Tam, Shisong Rong, Calvin C. P. Pang, Guy L. J. Chen, and Wai Kit Chu

Abstract

Glaucoma is a leading cause of irreversible blindness worldwide. Genetic studies in glaucoma provide evidences of genes and loci related to the disease development and insights of the pathogenesis in glaucoma. Gene mutations with strong associations to the disease were found in family linkage studies in which the glaucoma patients usually had early-onset and severe disease features. From these studies, several genes, such as *MYOC*, *OPTN*, *TBK1*, and *TIE2*, have been found to be related to glaucoma. On the other hand, sequence variants linked to common and late-onset forms of glaucoma were mainly discovered from genome-wide association studies (GWAS). Up to date, 15 GWAS have identified common variants for the risk of different types of glaucoma including primary open-angle glaucoma (POAG), primary angle-closure glaucoma (PACG), and exfoliation glaucoma (XFG). While many GWAS utilized East Asian cohorts as primary cohorts, the effects of the variants contributing to glaucoma susceptibility vary across ethnic groups due to differential genetic background. In this chapter, we focus on the genetics of different glaucoma forms in East Asian populations.

Keywords

Glaucoma · Genome-wide association study · East Asians

29.1 Introduction

Glaucoma, a leading cause of irreversible blindness worldwide, is a group of optic neuropathies with characteristic structural optic nerve fiber loss and functionally visual field defects. Glaucoma is more common and could progress more rapidly in the aging population [1]. Recent epidemiological evidences reported the prevalence of glaucoma was 3.54% for people from 40 to 80 years old worldwide. This prevalence is at a similar rate of 3.4% in Asia [2, 3]. Glaucoma is a heterogeneous disease including subtypes of different etiology, prevalence, genetic susceptibility, clinical manifestations, and treatment strategies.

S. Y. Lu · C. C. Y. Tham · P. O. S. Tam · C. C. P. Pang
W. K. Chu (✉)
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, China
e-mail: waikit@cuhk.edu.hk

S. Rong
Department of Ophthalmology, Massachusetts Eye and Ear, Harvard Medical School, Boston, MA, USA

G. L. J. Chen
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, China

Department of Ophthalmology and Visual Sciences, Prince of Wales Hospital, Hong Kong, China

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Based upon the anatomical structure of the ocular anterior segment, it can be divided into two major subgroups, open-angle (OAG) and closed-angle (ACG). Structural variations in the anterior chamber angle could affect the drainage rate of aqueous humor, fluid secreted from the ciliary body and removed through the trabecular meshwork and Schlemm's canal, leading to changes in the intraocular pressure (IOP) [4]. Elevated IOP is an important risk factor for most of the glaucoma types. Normal tension glaucoma (NTG), a form of open-angle glaucoma, however, can occur with no IOP elevation [5]. In addition, glaucoma can develop at young age. There is also congenital glaucoma that occurs in infancy essentially following the Mendelian inheritance. Congenital glaucoma is a rare disease, triggered by rare mutations with strong genetic effects, while later-onset glaucoma is influenced usually by common variants with weaker genetic effects [6]. Furthermore, genetic variations can not only influence the risks of primary glaucoma but also some cases of secondary glaucoma such as exfoliation glaucoma (XFG) [7].

Genetic factors contribute to a significant proportion of risk in glaucoma (Fig. 29.1). Different methodologies have been applied for investigation of glaucoma genetics including family linkage analysis, re-sequencing study, genome-wide association study (GWAS), and next-generation sequencing (NGS) [8–13]. Most glaucoma patients are non-symptomatic at the early stage. Therefore, early detection is a challenge for disease management. One important purpose of genetic studies is to identify individuals at risk by developing precise genetic screenings of high disease susceptibility. These genetic screenings will help the decisions on early intervention to prevent or slow down the disease progression. Understanding of genetic factors and biological mechanisms of different glaucomas provides information to devise new therapeutics to improve treatments and to develop gene-based therapies [14].

29.2 Pathophysiology of Glaucoma

The pathophysiology of glaucoma is complex and still not fully understood. Glaucomatous optic neuropathies are associated with progressive neuroretinal rim narrowing and effects on the optic cups. Retinal nerve fiber layer (RNFL)

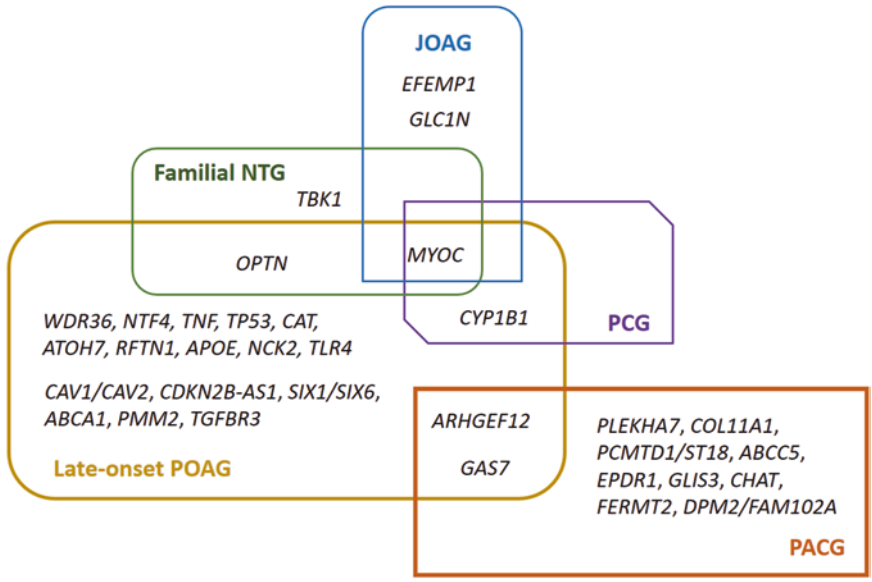

Fig. 29.1 Overlaps between the genes associated with one or more glaucoma subtypes in East Asians

thinning and arcuate visual field loss are common features of most glaucoma patients, indicating damages in the optic nerve head [15]. Optic nerve injury is generally considered as the dysfunction or loss of neurons such as retinal ganglion cells (RGCs). One of the putative causes for RGC death is axon injury by the displacement of lamina cribrosa, which is a sieve-like membrane for RGC axons to exit to the posterior part of the sclera [16]. Elevated IOP raises the pressure gradient through the lamina cribrosa. Deformation of this tissue causes damages to the optic nerve head and RGC axons [17]. In angle-closure glaucoma, structural disruptions affect positioning of the peripheral iris with respect to the trabecular meshwork and peripheral cornea, leading to obstruction of aqueous outflow and consequently elevation in IOP [4]. In open-angle glaucoma, although the anterior chamber angle is open, there are metabolic or structural derangements affecting drainage of aqueous humor, such as defects of mitochondrial function, reduction of endothelial cells, and abnormal accumulation of extracellular matrix components in the trabecular meshwork [18–20]. Elevated IOP could induce glaucoma through ischemia, oxidative stress, autophagy, excitotoxic signaling, and immune response [21–24]. However, the IOP of NTG patients is within the normal range which is usually defined as 10–21 mm Hg. Reduced ocular perfusion pressure, the net gradient between systemic blood and intraocular pressure, is also a risk factor for optic nerve damage and glaucoma [25, 26]. Perfusion of vasculature and resultant oxidative stress in the optic nerve might be associated with NTG pathophysiology [27].

29.3 Primary Congenital Glaucoma (PCG)

PCG is a serious childhood glaucoma usually developed before 3 years old, compared to a later-onset time in juvenile-onset primary open-angle glaucoma (JOAG). Prevalence of PCG varies widely across different ethnic populations, reportedly from 1/1250 in Middle East populations and South Asians to 1/70,000 in Caucasians [28]. High occurrence in the former populations is likely associated with consanguinity. PCG has been found to be associated with myocilin (*MYOC*) mutations, with Glu230Lys, Arg272Stop, and Ser313Phe reported in a Chinese study and p.Leu228Ser and p.Glu240Gly identified in a South Korean study [29, 30]. The most important gene for PCG is *CYP1B1* (cytochrome P450 family 1 subfamily B member (1), which accounts for most congenital glaucoma patients. *CYP1B1* mutations which specifically occur in East Asians have been reported. In a Japanese study, four *CYP1B1* mutations, Asp192Val, Ala330Phe, Val364Met, and Arg444Gln, were identified in PCG [31]. A Chinese study reported 11 novel *CYP1B1* mutations in PCG including g.3985C>G (p.Ile60Met), g.4089T>C (p.Val95Ala), g.4124C>G (p.Leu107Val), g.4157C>T (p.Pro118Ser), g.4206T>C (p.Phe134Ser), g.4413A>G (p.Asn203Ser), g.4664G>A (p.Ala287Ser), g.4677A>G (p.Asp291Gly), g.4761A>G (p.Asn319Ser), g.7925T>A (p.Val363Asp), and g.7940G>T (p.Arg368Leu) [29]. Subsequently, Kim et al. reported 11 mutations from 22 of 85 Korean PCG patients. In particular, two of these mutations, p.sGly329Ser and p.Val419Glyfs11Stop, were novel [30]. *CYP1B1* has been shown to form digenic interaction with *MYOC*, resulting in higher risk of glaucoma [32].

29.4 Familial Normal Tension Glaucoma (NTG)

Familial NTG is a rare and early-onset type of POAG, which has been reported to associate with mutations in *optineurin* (*OPTN*) and *TANK-binding kinase 1* (*TBK1*). *OPTN* Glu50Lys is the most frequent mutation identified in familial NTG in East Asians; several studies have identified *OPTN* mutations in early-onset NTG individual patients instead of NTG families [33]. In a Korean study, homozygous Met98Lys, homozygous Thr34Stop, heterozygous Arg271Cys, and combined heterozygous Thr34Stop with Arg545Gln mutations in *OPTN* were found in early-onset NTG patients. Moreover, a combined

heterozygous mutation Thr123Stop and Ile288Stop in *MYOC* in a female NTG patient was found [34]. Arg158Gln in *MYOC* was found from an early-onset NTG patient in Japan [35]. Susceptible loci in *TBK1* in familial NTG were first identified from three NTG pedigrees in the United States: *GGO-441* from an African-American family and GGA-*416* and *GGA-1159* from two Caucasian families. In the African-American family, three copy number variations (CNVs) within the *GLC1P* locus, located on chromosome 12q14, co-inherited with the patients. Interestingly, *TBK1* is located in the *GLC1P* locus and was expressed in ganglion cells, nerve fiber layer, and microvasculature of the human retina [36]. A subsequent study discovered a novel *TBK1* mutation, in locus *GGJ-414*, from a Japanese NTG pedigree [37]. As both OPTN and TBK1 are reported to involve in autophagy and NF-KB signaling, these pathways may play important roles in NTG [38].

29.5 Juvenile-Onset Primary Open-Angle Glaucoma (JOAG)

Primary open-angle glaucoma (POAG), characterized by normal anatomic configuration of the aqueous humor outflow pathway, is the most common form of glaucoma in the United States and Western Europe. Globally, the highest prevalence of POAG is in Africa, 4.20%, while PACG is in Asia (1.09%) [3]. JOAG is an early-onset form of POAG before the age of 40 years old. The first glaucoma gene locus, *GLC1A* on chromosome 1q24.3, was identified in a large Caucasian family [39]. The responsible gene in this locus for POAG was found to be trabecular meshwork-inducible glucocorticoid response (*TIGR*), which is also called *myocilin* (*MYOC*) [40].

29.5.1 MYOC

A number of studies have been conducted to investigate mutations in *MYOC* and other loci linked to JOAG and late-onset POAG in East Asians (Table 29.1). A Japanese study in 1997 detected a new mutation Pro370Leu in *MYOC* from a JOAG family. In this family, the father and the daughter were diagnosed of having glaucoma at 26 and 16 years old, respectively. Gly367Arg was identified in a female diagnosed with POAG at 45 years old from another Japanese family [41]. A Korean family study in 1999 reported two mutations Arg46Stop and Thr353Ile in *MYOC* in JOAG [42]. However, our study in southern Chinese in Hong Kong suggested that both the Arg46stop and Thr353Ile in *MYOC* could be found in both POAG patients and normal controls. Moreover, our study found a novel mutation, Arg91Stop in *MYOC*, in a late-onset POAG [9]. We also reported a Arg46stop truncation in *MYOC* in one JOAG patient, four late-onset POAG, and nine normal controls [43]. The mutation Ala363Thr was first reported in a mutation screening study in Japanese JOAG patients [35] and another missense *MYOC* mutation, Cys245Tyr, from a Chinese JOAG family in Hong Kong by our group [8]. The variant Arg46Stop was also reported by a Chinese JOAG study in Taiwan together with Val56Ala, c.604 + 228A > T in the intron, and c.1515 + 73G > C in the 3′-untranslated region of *MYOC* [44]. A nonsynonymous substitution Asp384Asn in *MYOC* was first identified from a northern Chinese JOAG family [45] and Pro254Arg from a JOAG family in Sichuan of Western China. Patients in this family suffered severe visual field loss and elevated IOP at 31 mm Hg in both eyes, with disease onset younger than 40 years of age [46]. Two combined heterozygous mutations (c.-83G > A and c.764 T > C, coding for Leu255Pro, and c.369C > T and c.864C > T, coding for Thr123 = and Ile288=, respectively) were found in two Korean JOAG patients [34]. Screening for sequence variations in the *MYOC* promoter did not show mutations in both HK Chinese and Korean POAG patients [47, 48]. These results imply that the modulation of MYOC expression might not affect glaucoma development. In addition, *MYOC* mutations were also identified in late-onset POAG (Glu300Lys, Tyr471Cys,

Table 29.1 Novel mutations/loci discovered from JOAG patients in East Asian

Gene (HGNC locus designation)	Chromosome	Location	Sequence alteration	Ethnic group	References
MYOC (GLC1A)	1q24.3	Exon	Pro370Arg	Japanese	[41]
		Exon	Gly367Arg	Japanese	[41]
		Exon	Arg46X	Korean	[42]
		Exon	Thr353Ile	Korean	[42]
		Exon	Ala363Thr	Japanese	[35]
		Exon	Cys245Tyr	Southern Chinese	[8]
		Exon	Val56Ala	Taiwan Chinese	[44]
		Intron	c.604 + 228A > T	Taiwan Chinese	[44]
		3′ UTR	c.1515 + 73G > C	Taiwan Chinese	[44]
		Exon	Asp384Asn	Northern Chinese	[50]
		Exon	Pro254Arg	Southern Chinese	[46]
		Exon	Leu255Pro	Korean	[34]
		Exon	Thr123=	Korean	[34]
		Exon	Ile288=	Korean	[34]
Unknown gene *(GLC1N)*	15q22-q24	No reported	No reported	Southern Chinese	[66]
EFEMP1 (GLC1H)	2p15-p16	No reported	No reported	Southern Chinese	[67]

Ile360Asn, Gly451Asn, Ala363The, Phe369Leu, and Thr448Pro) as well as familial NTG patients (Arg158Gln, and a combined mutation of Thr123= and Ile288=) from Hong Kong, Japan and Korea [9, 34, 35, 43, 49, 50, 51]. These results indicate that *MYOC* mutations also appeared in the late-onset POAG patients. Overall, *MYOC* mutations account for 2–4% of late-onset POAG [6].

MYOC encodes a secreted glycoprotein modulating different signaling pathways to regulate cell-cell junctions, cell-matrix adhesion, cytoskeletal network, and cell migration in adjacent cells [52–54]. *MYOC* mutations associating with glaucoma and increased IOP are considered to mainly affect the physiology in trabecular meshwork and the aqueous humor outflow pathway [55, 56]. However, changes in *MYOC* expressions were not associated with ocular hypertension and glaucoma development [57–59]. The misfolded mutant MYOC could trigger endoplasmic reticulum stress in trabecular meshwork cells to induce resistance in aqueous humor outflow and IOP elevation [60]. An animal study showed that reducing endoplasmic reticulum stress could be a potential therapeutic strategy for glaucoma patients with *MYOC* mutations [61].

29.5.2 Other Gene Loci

MYOC mutations account for 8–36% of JOAG in different reported studies. It is also induced by mutations in other genes and environmental factors [6]. Several POAG related loci, *GLC1H*, *GLC1J*, *GLC1K*, *GLC1M*, and *GLC1N*, were identified in JOAG patients. *GLC1J* (9q22) and *GLC1K* (20p12) are the only two JOAG associating loci reported in Caucasian studies [62]. Our previous work found *GLC1M*, 5q22.1-q32, was associated with autosomal dominant JOAG in a Philippine family [63]. *NRG2* and *SPARC* from the *GLC1M* locus were excluded to be the causal genes of JOAG [64, 65]. *GLC1N*, 15q22-q24, was identified in a Chinese JOAG family [66]. Genes in both *GLC1M* and *GLC1N* linked to JOAG are still unknown. *GLC1H*, 2p16.3-p15, was also reported to associate with JOAG in a Chinese family [67]. A mutation in *EFEMP1*

(c.418C > T, Arg140Trp) in this locus was identified by exome sequencing in an African-American family with adult-onset POAG [67, 68]. In addition, we found significant association of POAG with interactions between two mutations, *MYOC* (Thr353Ile) and *OPTN* (IVS15 + 10G > A), indicating polygenic etiology of glaucoma [69].

29.6 Adult-Onset Primary Open-Angle Glaucoma (POAG)

29.6.1 *MYOC, OPTN,* and *WDR36*

To identify mutations and common variants associated with POAG development, target genes or loci were explored in case-control studies by direct sequencing and genotyping. Some common variants and mutations associating with the early-onset POAG, such as *MYOC* and *OPTN*, contributed to the risks of developing adult-onset POAG in East Asians [9, 34, 35, 43, 49–51]. Candidate gene-based investigations of late-onset POAG have reported loci with differential allelic frequencies between POAG patients and healthy controls. The WD repeat domain 36 gene (*WDR36*), located in the *GLC1G* locus on chromosome 5q22.1, is related to POAG especially for high tension glaucoma (HTG). A study in southern Chinese identified one disease-predisposing mutation, Ile713Val in *WDR36* with a frequency of 3.7% in HTG patients. In addition, three SNPs (rs13153937, rs10038177, and rs11241095) were significantly associated with HTG, while no *WDR36* SNPs was associated with NTG or JOAG [70]. A genome-wide linkage scanning for IOP-related loci in Mongolian patients found an association between the *WDR36* locus and IOP regulation [71].

29.6.2 Other Associated Genes

Tumor necrosis factor (*TNF*), coding for a monocyte-derived cytotoxin that causes cell death, was first associated with POAG in a Chinese study [72]. SNP rs1800629 in *TNF* had significant association with HTG and SNP rs1042522 in tumor protein p53 (*TP53*) significantly associated with NTG in Hong Kong Chinese [73]. Both the *TNF* and *TP53* genes that play key roles in apoptosis and t are related to tumorigenesis. The neurotrophin-4 (*NTF4*) gene stimulated the receptor tropomyosin receptor kinase B on retinal ganglion cells and suppressed cell death [74–76]. Mutations of *NTF4* have been identified in POAG patients from Europe and Asia. The nonsynonymous variants, Cya7Tyr, Glu84Lys, Ala88Val, Arg90His, Arg206Trp, Arg206Gln, and Arg209Gly, were reported in a European study, while three disease-causing mutations, Leu113Ser, Gly157Ala, and Ala182Val, were reported in two Chinese studies [77–79]. The *CYP1B1* gene was first reported for primary congenital glaucoma. However, Pro93Ser, Arg259Cys, Ala295Thr, and Leu475Pro of *CYP1B1* were identified from POAG patients in a northern Chinese study [80]. Oxidative stress is proposed to cause cellular damage and optic neuropathy. Enzymes involving in anti-oxidation have been investigated in POAG. A study in Sichuan, Western China, genotyped the *catalase* (*CAT*) gene, which encodes an antioxidant enzyme secreted into the aqueous humor, and found SNP rs769217 in *CAT* associated with POAG [81]. Studies on loci identified from GWAS related to glaucoma endophenotype found that SNPs in *atonal homolog 7* (*ATOH7*) and *raftlin lipid raft linker 1* (*RFTN1*) were associated with increased risks of POAG in Hong Kong Chinese [82].

NTG is affected by genetic factors other than those for HTG. Apolipoprotein E (*APOE*), located in 19q13.2, is related to Alzheimer's disease [83, 84]. *APOE* is involved in lipid transport in the metabolism of neuronal cell membrane. Several SNPs in *APOE* were associated with POAG in Caucasians [85, 86]. Our two studies in Hong Kong Chinese confirmed the association between the *APOE* epsilon 4 allele and NTG and also indicated the interaction of *APOE* and glaucoma genes *MYOC* and *OPTN* [69, 87]. *NCK2*, encoding the NCK adaptor protein 2, was linked to NTG in a Japanese cohort [88]. Another Japanese study found three SNPs in Toll-like

receptor 4 (*TLR4*), rs10759930, rs1927914, and rs7037117, significantly affected risks for NTG [89]. TLR4 participates in the activation of innate and adaptive immune responses. However, the association could not be replicated in a Korean study. Furthermore, only SNP rs7037117 in *TLR4* was associated with NTG in a recessive model in southern Chinese [90, 91].

29.6.3 GWAS for POAG

Apart from the abovementioned genetic factors, there are also sequence alterations in other genes reported in POAG. However, many of them only had weak association and could not be replicated in later studies, probably due to small sample sizes and ethnic differences between the study populations. GWAS is a powerful genomic technology to discover disease-related loci. So far, 9 GWAS studies in POAG have been reported, and SNPs in 15 genes/loci reached genome-wide significant level ($P \leq 5 \times 10^{-8}$). These loci are near or located in *CAV1/CAV2*, *TMCO1*, *CDKN2B-AS1*, *SIX1/SIX6*, *8q22*, *ABCA1*, *AFAP1*, *GMDS*, *ARHGEF12*, *PMM2*, *TGFBR3*, *TXNRD2*, *ATXN2*, *FOXC1*, and *GAS7* [92–100]. Among these nine GWAS, four of them included East Asian cohorts (Table 29.2) [92, 95, 97, 98]. Variants in *CDKN2B-AS1* were consistently detected in GWAS in different populations with top-tier statistical significances. Moreover, it showed stronger relationship to NTG compared to other types of POAG [94, 100]. Human and animal studies have shown expressions of *CDKN2B-AS1* in the inner retinal nuclear layer, ganglion cell layer, and trabecular meshwork. Variants in *CDKN2B-AS1* were shown to increase RGC death [101, 102]. Another GWAS showed that *FNDC3B* rs4894796 was in association with POAG in Asians (OR = 0.89, $P = 7.93 \times 10^{-8}$) but not in Caucasians (OR = 0.99, P = 0.71) [98]. *FNDC3B* is expressed in trabecular meshwork, optic disc, and optic nerve. Another *FNDC3B* SNP rs6445055 was significantly associated with IOP in a GWAS [12]. These evidences implied that the *FNDC3B* gene is involved in regulating aqueous humor outflow and IOP. Genes identified from GWAS have provided insights of biological pathways that might be involved in pathophysiology of POAG. For instance, identifications of *CAV1/CAV2* and *ABCA1* suggest the roles of lipid metabolism in POAG, while the discovery of *PMM2* implies that the fructose and mannose metabolism is related to POAG [103–107].

29.7 Primary Angle-Closure Glaucoma (PACG)

PACG is characterized as a condition of acutely or chronically raised IOP due to an abnormal configuration of the anterior chamber angle. The closed angle acts as a physical barrier to the aqueous humor outflow. Extremely high IOP is a feature of PACG to trigger optic nerve degeneration. Asians have increased predisposition to PACG compared to Europeans and Africans. More than 80% of PACG cases are in Asia [108]. GWAS has identified most of the known loci for PACG, two GWAS in PACG and one in anterior chamber depth (ACD). These studies reported that there was one SNP associated with both PACG and ACD and nine genes/loci were identified, including *PLEKHA7*, *COL11A1*, *PCMTD1/ST18*, *ABCC5*, *EPDR1*, *GLIS3*, *CHAT*, *FERMT2*, and *DPM2/FAM102A* [11, 109, 110]. In addition, genotype analysis of POAG-associated SNPs in case and control cohorts suggested that only two SNPs, rs2276035 at *ARHGEF12* and rs12150284 at *GAS7*, were significantly associated with PACG [110]. The first PACG GWAS and ACD GWAS discovered genotypic associations in Asian, while the latter PACG GWAS involved cohorts from 24 countries across five continents. Most genes reported from these GWAS were found to be expressed in human ocular tissues except *PCMTD1* and *ST18*. In particular, mRNA of *EPDR1*, *GLIS3*, *FERMT2*, *DPM2*, and *FAM102A* were found in human iris, ciliary body, and trabecular meshwork [4]. Expressions of these genes in the ocular anterior segment tissues indicated possible effects on the configuration of the anterior chamber angle and the drainage pathway of the aqueous humor. Additionally,

Table 29.2 POAG loci discovered by GWAS involving East Asian

Gene	SNP	Discovery cohort			Replication cohort			References
		Country	Ethnic	Size (case vs control)	Country	Ethnic	Size (case vs control)	
CAV1/CAV2	rs4236601	Iceland	Caucasian	1263 vs 34,877	Sweden, UK, Australia, China	Caucasian; Asian	2474 vs 2644	[92]
CDKN2B	rs1063192	Japan	Japanese	1394 vs 6599	Japan	Japanese	1802 vs 7212	[95]
SIX1/SIX6	rs10483727							
ABCA1	rs2487032	Southern China	Chinese	1007 vs 1009	China and Singapore	Chinese	1899 vs 4965	[97]
PMM2	rs3785176							
TGFBR3	rs1192415	Multiple	Multiethnic	3504 vs 9746	Multiple	Multiethnic	9173 vs 26,780	[98]
FNDC3B	rs4894796							

PLEKHA7, *EPDR1*, and *FERMT2* played roles in cell adhesion and junction. Abnormal function of these gene products might influence the tightness of cell adhesion in anterior segment tissues, leading to changes in the anatomic structure in the chamber angle [109, 110]. SNPs in *ABCC5* did not show genome-wide association with PACG but ACD. Genotype-phenotype analyses showed that SNPs in *ABCC5* were significantly associated with PACG in a Chinese study [11, 111]. Other candidate genes reported by PACG GWAS included *COL11A1*. It is involved in fibrillogenesis and extracellular matrix binding that may have potential to alter the tissue structure [112]. *CHAT* participates in neurotransmitter biosynthetic processes and *DPM2* in biosynthesis of dolichol-phosphate mannose [113, 114]. Mutations of *GLIS3* have been reported in congenital diabetes and common *GLIS3* SNPs also related to diabetes [115–117]. Finally, a meta-analysis revealed seven polymorphisms in five genes/loci associated with PACG: rs17427817 and rs5745718 at *HGF*, rs1043618 at *HSP70*, rs2510143 and rs3814762 at *MFRP*, rs3918249 at *MMP9*, and rs7830 at *NOS3* [118]. In summary, the candidate genes identified from both GWAS and meta-analysis could suggest new target genes for further investigation in biological functions and exploration of underlying mechanism of PACG pathophysiology.

29.8 Exfoliation Syndrome (XFS) and Glaucoma

XFS is an age-related disease characterized by the deposition of abnormal amyloid-like fibers in the extracellular matrix in different tissues primarily in the eyes. Aggregates of pigments and fibrillar materials in the anterior segment tissues such as ciliary body, trabecular meshwork inner surface, and Schlemm's canal would induce blockage of aqueous outflow and lead to increased IOP, resulting in the development of secondary glaucoma [119]. Epidemiology studies showed that about one fourth of XFS patients had elevated IOP and 30% of them developed exfoliation glaucoma (XFG), which is the most common identifiable type of open-angle glaucoma worldwide [120–122]. XFG individuals have higher mean IOP, more aggressive visual field loss, and worse prognosis with resistance to IOP-lowering medications compared with POAG patients. The mechanism underlying abnormal aggregation of extracellular materials that trigger XFS is still not fully understood, but likely involved both genetic and environmental factors [123].

Unlike POAG, there is no candidate gene or locus that has been identified for XFS/XFG from familial studies. The first gene contributing significant risk for XFS was reported by a GWAS in which the primary cohort contained 75 cases and 14,470 healthy controls from Iceland. The gene *LOXL1*, encoding lysyl oxidase homolog 1, and SNPs rs3825942, rs1048661, and rs2165241 in this region were genome-wide associated with XFS [124]. The associations have been replicated in other Caucasian populations and different ethnic groups including Chinese, Japanese, and Indian [7, 125–129]. In addition, there was no association between *LOXL1* locus and POAG in all reported populations [130–132]. However, there is some evidence showing allelic reversal of rs3825942 on XFS in South Africans [133]. This allelic reversal was further supported by a subsequent GWAS, in which the primary Japanese cohort showed the A allele of rs3825942 with OR = 9.87 ($P = 2.13 \times 10^{-217}$), while the OR of this allele in non-Japanese (Caucasian, African, Indian, and Chinese) is 0.49 ($P = 2.35 \times 10^{-31}$). Another locus, rs4926244, in *CACNA1A* was also reported with significant association with XFS by this GWAS. These findings implicated a complex mechanism of allelic effects at the *LOXL1* locus and other gene factors in the pathogenesis of XFS [134]. Furthermore, a recent GWAS with a larger sample size including 13,838 cases and 110,275 controls from 24 countries across 6 continents confirmed the previous associations and found 5 more XFS loci (*POMP*, *TMEM136*, *AGPAT1*, *RBMS3*, and near *SEMA6A*) [135]. These studies expanded the spectrum of gene candidates involved in the biological pathways and processes that contribute to XFS/XFG development.

29.9 Conclusive Remarks

In summary, a number of genes and loci were identified by genetic studies of glaucoma patients in East Asia. Most of them are specific to only one type of glaucoma, while several genes play roles in multiple disease forms, such as *MYOC* in POAG and *ARHGEF12* and *GAS7* in both late-onset POAG and PACG (Fig. 29.1). This information suggests specific targets and relevant biological pathways to account for pathogenic mechanisms in glaucoma. Better understanding of genetic and biological architecture of glaucoma is required for the development of early diagnosis and potential novel treatments for glaucoma.

Compliance with Ethical Requirements Shiyao Lu, Clement C.Y. Tham, Pancy O.S. Tam, Shisong Rong, Calvin C.P. Pang, Guy L.J. Chen, and Wai Kit Chu declare that they have no conflict of interest. No human or animal studies were performed by the authors for this article.

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Quantitative Trait for Glaucoma

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Sarangapani Sripriya, Ferdina Sharmila, Suganya Kandeepan, and Ronnie George

Abstract

Dissecting the genetic architecture of complex or multifactorial disorders always demanded different strategies in addition to the classical gene mapping methods, due to the high level of clinical and genetic heterogeneity. An endophenotype or intermediate phenotype is a measurable trait with a strong genetic component (determined by heritability and twin studies) which associates with a disease. Endophenotypes for POAG include intraocular pressure (IOP), central corneal thickness (CCT), cup area (CA), vertical cup-disc ratio (VCDR) and disc area (DA). The loci fo these genetic determinants are called as quantitative trait loci (QTLs). Identifying the QTLs for the clinical risk factors is considered as an effective strategy for mapping the genes contributing to the overall disease pathology. We review the various ocular QTs and the insights gained into the pathogenesis of glaucoma through QTL mapping.

S. Sripriya · F. Sharmila · S. Kandeepan
SNONGC Department of Genetics and Molecular Biology, Vision Research Foundation, Chennai, India

R. George (✉)
Smt. Jadhavbai Nathamal Singhvee Glaucoma Services, Medical Research Foundation, Chennai, India
e-mail: drrg@snmail.org

Keywords

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30.1 Quantitative Traits or Endophenotypes

An endophenotype, also known as intermediate phenotype or a quantitative trait, is a quantifiable biological trait that is heritable and influences the course of primary disease. These traits are considered important in understanding the genetics of the primary disease as they contribute to the varying disease severity observed for a disease in question and thus could potentially explain the heterogeneity in the disease presentation [1]. In single gene disorders, whether they are dominant or recessive, the phenotype is discrete and qualitative, whereas, in complex diseases, the phenotype is heterogeneous which is accounted due to shift in the quantifiable risk predisposing clinical features. Many human quantitative trait loci (QTL) have been shown to exhibit additive or recessive inheritance patterns, indicating the contribution from multiple genes (or alleles) for phenotypic expression.

Co-segregation at a single genetic locus or at numerous interacting loci could exhibit a cumulative effect and result in a clinical presentation. Such additive effects on susceptibility to complex traits are over-represented in communities

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with higher frequency of consanguineous marriage because siblings in consanguineous families would inherit two copies of the same allele or set of alleles, contributing to the trait. This could manifest as a greater variation in the magnitude of a quantitative trait because of the additive effects of the allele(s).

These endophenotypes however individually exert only small effects but collectively result in complex diseases. An overt expression of the combined effects of these factors which crosses a hypothetical threshold manifests as a disease such as in schizophrenia, bipolar disorder, depression, etc. For example, deficits in working memory are known to increase the risk for schizophrenia and are considered as an endophenotype for the disease. At the molecular level, specific alleles associated with the risk of dopaminergic dysregulation in the prefrontal cortex modulate effect on working memory which subsequently increases the risk for schizophrenia. Thus identification of quantitative traits and their genetic loci (called quantitative trait loci) is considered as an alternative to traditional disease association studies, especially in complex diseases. With respect to neurological disorders, as listed above, endophenotypes are selected for further genetic analysis when they exhibit specific properties [2]. Some of the requisites are that the endophenotypes should be heritable (heritability defines the proportion of the phenotype variance due to genetic components) and play a causal role in the disorder, the effect size of a particular gene should be greater in relation to the endophenotype than to the clinical syndrome and they should vary continuously in the general population. Grouping patients based on the endophenotypes is considered to be effective for mapping the linked genetic loci and the genes involved.

30.2 From Endophenotypes to Disease

Genetic predisposition has been one of the major risk factors for glaucoma, and the discovery of genes predisposing to glaucoma through family-based studies has been the initial approach to understand the molecular events in the pathology of the disease. The search for glaucoma genes have been through linkage studies which focussed on families affected by Mendelian forms of the disease. The loci mapped to POAG from GLCA to GLCP did not explain a significant proportion of the genetic basis for POAG. Out of the regions mapped, the *MYOC*, *OPTN* and *WDR36* genes were widely studied across various populations in family-based studies. *MYOC*-associated glaucoma accounted for 3–5% of POAG cases worldwide with more than 100 reported mutations worldwide, some of which were population specific (Gln368Stop, Arg46Stop, Gln48His).

OPTN (GLC1E, 10p13), the other gene studied more frequently across various populations, has a mutation frequency of 16.7% in hereditary forms of normal tension glaucoma (NTG) and 1–2% of POAG cases. Specific mutations like E50K have been frequently observed in POAG patients and are also correlated with various clinical features. POAG patients harbouring the E50K mutation have an earlier age of onset, a more advanced optic nerve cupping and a more frequent need for surgical intervention [3]. In the Indian context, this mutation was not observed, and we have reported the possible role of SNPs rather than mutations in POAG patients. The other gene observed in <1% of normal tension POAG cases is TBK1 (GLC1P, 12q14) that codes for serine/threonine kinase with a major role in the regulation of inflammatory responses [4]. TBK1-OPTN protein is shown to mediate the interaction between mitophagy and immune response [5]. This has been shown to have a potential therapeutic implication for neurodegenerative diseases like glaucoma. Yet, these details were not sufficed to explain the pathology of glaucoma.

The WD repeat domain 36 (*WDR36*: GLC1G, 5q22) gene codes for a nucleolar protein implicated in the maturation of 18 s rRNA. The observed frequency of mutations ranges from 1.6% to 17% of POAG patients [6] that was however not replicated in subsequent studies. Similar to the observation of OPTN gene variants in the Indian population, the *WDR36* variants rather than mutations were suggested to be associated with the risk of POAG. Mutations in *NTF4* gene (GLC1O, 19q13.33) contributed to 1.7% of

POAG patients of European origin [7] that has not been replicated in other populations (India, China). The other gene that was mapped in family-based studies and later replicated in case-control studies is ASB10 (GLC1F, 7q36). Besides, copy number variations in *TBK1*, genomic duplications (in *CDKN2B*-AS1, near *GAS7* and *TMCO1*) and deletions (in *SIX6* and *ATOH7*) have been reported in POAG cases.

As discussed above, the genes identified through family-based studies and then replicated through case-control studies accounted to a frequency of 1–5% of POAG in the general population. The limitations in family based studies such as the unavailability of multiple members affected large families, sample size and technological limitations restricted the understanding of the molecular pathology of POAG in earlier phase of glaucoma research. Subsequent technological innovations, clinical phenotype standardisation and recognising the need for a consortium-based approach gave a new dimension to the genetic studies in POAG. The utility of classical genetics that shifted to genome-wide association studies (GWAS) through case-control study designs combined with a consortium-based approach proved effective in better understanding the genetics of POAG. In addition to disease associations, studies on the possible association of the genetic variants with the various clinically measurable risk factors for glaucoma (QTs or quantitative traits) are being studied across various population towards understanding of the glaucoma genetics. Identification of genetic factors for QTs that are highly heritable with significant variations amidst the general population (e.g. IOP, CCT, optic nerve parameters) is useful in mapping the genes contributing to the overall disease pathology. This strategy based on endophenotypes to map glaucoma genes is based on ranking individuals based on the variable risk traits rather than as patients and controls.

30.3 Ocular Quantitative Traits

Refraction parameters, anterior chamber depth, axial length, optic disc parameters, central corneal thickness, corneal curvature and intraocular pressure are some of the quantitative traits of the eye of which the optic disc parameters, CCT and IOP are well studied for their association with POAG.

Axial Length (AL) Axial length (AL) of the eye is an important refractive parameter which represents the length between the anterior and posterior poles of the eye. It is determined by the combination of anterior chamber depth, lens thickness and posterior chamber depth. AL increases most rapidly in infancy with growth slowing by adolescence and remains constant in adulthood. A study on 148 normal eyes of 79 patients ranging from premature newborns to 36-year-old adults showed that the mean AL measured 16.8 mm in a full-term newborn baby and 23.6 mm in an adult [8]. This increase with age causes a shift in refraction in the eye from hyperopia towards myopia. The final refractive state of the eye is a result of the combined changes in lens and corneal curvature. A 1 mm elongation of AL without these compensations is equivalent to a myopic shift (AL > 28 mm) of −2 or −2.5 dpt, thereby establishing the fact that the components of the visual system are in close interaction with one another during the entire maturation process. Heritability of axial length ranges from 0.89 to 0.94 [9].

Intraocular Pressure (IOP) Aqueous humour formed by the ciliary body is drained primarily through the trabecular outflow pathways, which includes the trabecular meshwork, the juxtacanalicular connective tissue, the endothelial lining of Schlemm's canal and the collecting channels and the aqueous veins [10]. IOP depends on the rate at which the aqueous humour is drained out and in normal cases the rate of production equals the rate of removal. When the rate of removal is less than the production, it starts to accumulate, leading to increase in IOP. Thus elevated IOP is the major risk factor of glaucoma which affects the retinal ganglion cells leading to axonal degeneration, abnormal optic nerve head appearance and finally blindness. IOP shows circadian variations and fluctuation and remains as the only modifiable and hence treatable risk factor for glaucoma. In the general population, elevated

IOP (over the upper normative limit of 21 mmHg) is not always accompanied by glaucomatous damage. While those with elevated IOP are more likely to have glaucoma, glaucomatous changes can occur with IOP in the physiological range too (normal tension glaucoma). The heritability estimates for IOP varies from 0.29 to 0.50. Short- and long-term fluctuations in IOP, non genetic factors probably accounted for its lower heritability [11].

Charlesworth et al. mapped the 10q22 as the locus for maximum IOP [12]. Interestingly the same region has been associated with systemic hypertension in a Japanese population. Expression QT analyses in UK twins identified a new locus *FAM125B* – a membrane complex involved in vesicular trafficking process [13]. Another GWAS in Australian cohort and later replicated in a UK cohort showed evidence of association with 7p21 near GLCCI1 and ICA1 [14]. A study by van Koolwijk et al. revealed an association with *GAS7* and *TMCO1* on chromosomes 17p13.1 and 1q24.1, respectively [15]. GWAS in subjects of European ancestry also identified significant associations with IOP at *TMCO1* (rs7518099-G, $p = 8.0 \times 10^{-8}$) and with other common variants in multiple genomic regions (CDKN2B-AS1, GAS7, CAV1/CAV2 and SIX1/SIX6) in regulating IOP [16]. Other loci found to be associated with IOP so far include *FNDC3B*, *ABCA1*, *ABO*, *11p11.2* and *ARHGEF12* [17, 18].

Optic Disc Parameters Characteristic changes on the optic nerve head are an important feature of glaucoma. Optic disc area and vertical cup-disc ratio (VCDR) are important quantitative traits that are altered in glaucoma. VCDR have been a longstanding method of documenting disc change in glaucoma and can be influenced by the optic disc size/area. In addition there exist racial variations in optic disc size. Familial aggregation for cup-disc ratio was initially reported through extended family studies by Armaly et al., in 2016. The correlation coefficient of vertical CDR was 0.25 in siblings and 0.24 in parents and children, as reported by Beaver Dam Eye study. Heritability of the ratio was estimated as 0.48 according to the parent-child correlation and 0.56 based on the data from siblings of the population-based Salisbury Eye Evaluation [19]. In a large twin study conducted in 1114 twins, inclusive of both mono and dizygotic twins revealed an intraclass correlation coefficients of 0.79 for disc area, 0.83 for cup area and 0.80 for cup-disc area ratio in MZ pairs and 0.30, 0.37 and 0.35, respectively, in DZ pairs. An additive genetic effect was also observed for disc area, cup area and cup-disc area ratio (77.3%, 82.7% and 78.6%, respectively) and thus concluded that 80% of these phenotype variances are genetically determined.

The loci associated with these parameters by GWAS-based studies included *ATOH7*, *CDC7/TGFBR3* and *SALL1*, *CARD10* for the optic disc area, and *CDKN2B*, *SIX1*, *SCYL1/LTBP3*, *SIX6*, *CHEK2* and *DCLK1*, in addition to *ATOH7*, for the VCDR [20–24]. Of the genes listed above, *ATOH7*, *CDKN2B* and *SIX1* are replicated for the association with POAG in other cohorts like the Rotterdam Eye Study with the Twin UK study [25] with borderline associations for *CDC7/TGFBR3* and *SALL4* (both $p = 0.04$). *CARD10* was not found to be associated with African-Caribbean POAG cases [14], whereas *CHEK2* was reported to be associated with VCDR and high tension glaucoma among the Japanese [26] but not in Europeans [27]. Multiple studies have provided strong evidence of association of *ATOH7*, *CDKN2B*(*-AS1*) and *SIX1*/*SIX6* with POAG [14, 25–30].

***ATOH7*, Atonal Homolog of Drosophila Gene (10q21.3)** ATOH is the atonal homolog of drosophila, named for its chordotonal stretch receptor mutant phenotype [31]. It is a basic proneural helix-loop-helix (bHLH) transcription factor expressed in the early retinal progenitors. This has been shown to be essential for the initiation of neurogenesis and production of retinal ganglion cells (RGCs) [32]. Defects in the *ATOH7* gene affect the RGC number, fate and differentiation of the other retinal cells [33, 34]. GWAS in European, Australian, Caucasians and Indian populations have shown association of rs1900004, rs3858145,

rs17231602 and rs4746741 with ODA [22–24, 30, 35]. Kamron et al. have documented that homozygous mutations (p.E49V and p. P18RfsX69) in this gene play a role in the development of both the anterior and the posterior chamber of the eye and are important in retinal vascular development and hyaloid regression [36].

SIX1/SIX6 **Gene (14q23)** These homeoprotein members belong to the SIX/*sine oculis* family of homeobox transcription factors. The *SIX1*/*SIX6* gene complex has been significantly associated with POAG in several GWAS. It was observed that *SIX6* missense variants deregulated its expression and affected its normal function in controlling retinal progenitor cell proliferation during eye development, hence influencing susceptibility to POAG [37]. Functionally this protein is involved in the regulation of retinal progenitor cell proliferation during eye development. Animal models have shown retinal hypoplasia, reduction in eye size and reduction in eye volume of the optic nerve [38–40].

CDC7/TGFBR3 **Gene (1p22)** Transforming growth factor (TGF) is a multifunctional cytokine which modulates the development of many tissue and repair processes by binding to its receptor *TGFBR3* [37]. This gene has been implicated in POAG development by its association with VCDR (rs1063192) in GWAS. Functionally it is known to interact with *CDKN2A*, another gene known to be associated with VCDR [14, 40, 41].

CDNK2B **Gene (9p21)** This gene is a member of the family of cyclin-dependent kinase (CDK) inhibitors which plays a role in cell cycle regulation, influencing the proliferation/differentiation balance. GWAS has shown association with VCDR in various populations [30, 42]. The protein is involved in the pathogenesis of optic nerve degeneration and POAG.

CARD10 **Gene (22q 13.1)** This protein is involved in the regulation of caspase activation and apoptosis. It activates NF-kappaB, a well-characterised transcription factor involved in signalling pathway [24, 35]. Variations in this gene have been proposed to influence the ODA. Associations between *CARD10* and ODA [24] have been reported in Asian and Singaporean cohort.

Central Corneal Thickness Corneal biomechanical properties are shown to influence intraocular pressure (IOP) measurements that include central corneal thickness (CCT) and corneal hysteresis (CH), corneal resistance factor (CRF) and corneal-compensated intraocular pressure (IOPcc). Studies like the Ocular Hypertension Treatment Study (OHTS) and European Glaucoma Prevention Study have revealed that CCT is an important risk factor associated with glaucoma. Familial correlation studies (Eskimos), heritability study based on twin studies, have demonstrated strong heritability estimates for CCT ranging from 0.6 to 0.7 till 0.95 among different ethnic groups [43]. Glaucoma progression was shown to be twofold higher in the eyes with thinner central corneas. In certain studies, CH has been shown to be associated with the diagnosis and risk of progression of POAG. CCT could be an independent risk factor for POAG apart from other factors; hence it has been proposed as an intermediate trait or endophenotype for POAG [44]. A recent study from the International Glaucoma Genetics Consortium (IGGC) identified 16 loci significantly associated with CCT [45]. Pathway analyses suggested that collagen and extracellular matrix pathways are important regulators of CCT. Genes associated include *ZNF469*, *FOXO1* and *FNDC3B* [46], *COL5A* and *COL8A2* [47, 48] and *AKAP13* [49] in both Caucasian (GWAS by Gutenberg and Rotterdam study) and Asian cohorts [50]. Quantitative analysis of CCT and a subsequent analysis of POAG revealed SNPs in two cell adhesion molecules, NTM and CNTNAP4, that may increase POAG susceptibility in a subset of cases [51]. Analysis of genes controlling CCT in multi-ethnic Indians

shows strong evidence of association at four novel loci: *IBTK* on chromosome 6q14.1, *CHSY1* on chromosome 15q26.3 and intergenic regions on chromosomes 7q11.2 and 9p23 [49]. A study in Caucasians identified a missense mutation in *COL8A1* associated with thin CCT [52]. Segmental chromosomal duplications or deletions encompassing FOXC1 were associated with increased CCT in families with early-onset glaucoma phenotypes [53].

Zinc Finger Nuclease (*ZNF469*) Gene (16q24) The protein functions either as a nuclear transcription factor or as an extranuclear regulatory molecule which is involved in synthesis, organisation and maintenance of collagen fibres [54]. Mutations in this gene have been associated with brittle corneal syndrome. SNPs near/in *ZNF469* (MIM 612078) and fork head transcription factor (*FOXO1*, MIM 136533) [55] have been associated with CCT, an important endophenotype of POAG in European and Asian cohort. This observation strongly supports *ZNF469* as a QTL for CCT [47].

Quantitative trait loci analysis in glaucoma by GWAS has identified significant associations for the various endophenotypes associated with glaucoma. In addition to this, a potential interaction specifying pathways linked to these genes in POAG is reported. These include pathways involved in extracellular matrix metabolism (ECM) regulating the CCT, signalling pathway related to ocular development and IOP. These pathways include transforming growth factor-β (TGF-β) signalling, tumour necrosis factor α (TNF-α) signalling, etc [56]. In the current context, our understanding on the endophenotypes and their role in normal and disease pathology is expected to expand with the advent of high-throughput next-generation sequencing technologies, reference genomes and different strategies that could range from population-based studies to inbred/consanguineous family-based studies which specifically increase the power of the study. Since glaucoma is an age-related disease that occurs later in life, traditional pedigree analysis has limited value given that the disease may not be manifested in younger individuals. Identifying genetic biomarkers for disease-associated endophenotypes addresses these limitations.

Compliance with Ethical Requirements This article does not contain any studies with human participants performed by any of the authors. This article does not contain any studies with animals performed by any of the authors. Sarangapani Sripriya, Ferdina Sharmila, Suganya Kandeepan and Ronnie George declare that they have no conflict of interest.

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Genetics of Exfoliation Syndrome in Asians

31

Prakadeeswari Gopalakrishnan, Aravind Haripriya, Banushree Ratukondla, and Periasamy Sundaresan

Abstract

Exfoliation syndrome (XFS) is common, age-related, and systemic microfibrillopathy. XFS is also a major recognizable cause of secondary open-angle glaucoma and blindness worldwide. It is considered to be a connective tissue disorder of extracellular matrix (ECM) that targets the tissues of the anterior eye segment through abnormal production and excessive accumulation of the fibrillary-flaky white-grayish material. The etiopathogenesis of XFS is still obscure, ten decades of research with XFS from the period of its first description. On the other hand, many explanations were made with both genetic and nongenetic factors, which are believed to be one among the causal mechanisms for XFS. This chapter provides evidence regarding the well-studied genetic, nongenetic, and other associated risk factor-based argument which enriches our understanding of this complex inherited disorder for future research.

P. Gopalakrishnan · P. Sundaresan (✉)
Department of Genetics, Aravind Medical Research Foundation, Dr G. Venkataswamy Eye Research Institute, Madurai, India
e-mail: sundar@aravind.org

A. Haripriya
Intraocular Lens and Cataract Clinic, Aravind Eye Hospital, Madurai, India

B. Ratukondla
Aravind Eye Hospital, Madurai, India

Keywords

XFS · SNP · Genetics · GWAS

Abbreviations

BIRC6	Baculoviral IAP repeat-containing 6
CACNA1A	Calcium voltage-gated channel subunit alpha 1 A
CAD	Coronary artery disease
CLU	Clusterin
CNTNAP2	Contactin-associated protein-like 2
ECM	Extracellular matrix
GST	Glutathione S-transferase
GWAS	Genome-wide association study
HLA	Human leukocyte antigens
IncRNA	Long noncoding RNA
IOP	Intraocular pressure
LOXL1	Lysyl oxidase-like 1
LOXL1-AS1	LOXL1 antisense RNA
MMPs	Matrix metalloproteinases
PEXS	Pseudoexfoliation syndrome
POAG	Primary open-angle glaucoma
SNPs	Single nucleotide polymorphisms
TM	Trabecular meshwork
TNF-α	Tumor necrosis factor-α
XFG	Exfoliation glaucoma
XFM	Exfoliation material
XFS	Exfoliation syndrome

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31.1 Introduction

Exfoliation syndrome (XFS) is a late-onset and conformational disorder of fibrillin, causing progressive elastotic degeneration and perhaps associated systemically with cerebrovascular and cardiovascular diseases [1]. It is a well-recognized risk factor for secondary glaucoma and known as a major reason for the irreversible blindness worldwide. Ritch et al. have estimated that around 60–70 million people worldwide get affected with XFS [2]. It is clinically characterized by the deposition of the fibrillary-flaky white-grayish small dandruff-like material over the different ocular tissues of the anterior segment including anterior lens capsule, lens zonules, iris, trabecular meshwork (TM), cornea, ciliary body, and the lamina cribrosa of the optic nerve [3, 4]. This deposition of the exfoliation material is not just limited to the eye which also occurs in non-ocular tissues like blood vessels and elastic connective tissues of the lung, kidney, liver, gallbladder, and cerebral meninges [5].

31.1.1 Discovery of Exfoliation Syndrome and Origin of the Term "Pseudoexfoliation"

Over a hundred years ago, John G. Lindberg, Finnish ophthalmologist, was first to describe the clinical condition which is now known as exfoliation syndrome (XFS) or pseudoexfoliation syndrome (PEXS). He observed and described it as grayish flakes and fringes at the pupillary border and noted the strange material formed a membrane on the anterior lens surface in senile cataract patients which was published as a thesis at the University of Helsinki in 1917 and translated in English later [6]. He also elucidated that this new phenomenon was shown to be common in both cataract patients and non-cataractous individuals above 55 years, and it was observed in 50% of the individuals with glaucoma. XFS was more prevalent with advancing age, which is an important decisive factor [7]. In 1922, Elschnig described "true exfoliation of the lens capsule," a rare disorder in which anterior layer of lens capsule delaminates, appears to be thin fluttering membrane in the anterior eye segment in glassblowers exposed to intensely hot and open fires [8, 9]. A Swiss ophthalmologist, Alfred Vogt, called this exfoliation of lens capsule as "glaucoma capsulare (capsular glaucoma)" [10]. Further, Georgiana Dvorak-Theobald an ocular pathologist noticed the precipitates and accretions of those unknown molecules around the anterior eye segment and proposed the term called "pseudoexfoliation." She distinguished it from "true exfoliation" of the lens capsule by pointing out their differences [11].

31.2 Worldwide Epidemiology of the Exfoliation Syndrome and Asian Perspective

The prevalence of the XFS is more predominantly observed in elderly people after their fourth decade. However, the distribution of XFS varies between the different ethnic populations [12]. This wide-ranging frequency of the XFS is evidenced by many studies including India (South India), 3.8% [13]; India (Tamil Nadu), 6.0% [14]; India (Central India), 0.95% [15]; India (Western India), 30% [16]; India (Andhra Pradesh), 3.01% [17], 0.6% [18], and 10.1% [19]; Nepal (Gurungs), 8.2%; Nepal (Tamangs), 0.3% [20]; Myanmar, 3.4% [21]; Kashmir, 26.32% [22]; Japan, 3.4% [23]; Republic of China, 2.38% [24]; Hong Kong (Chinese), 0.4% [25]; Singapore (Chinese), 0.2% [26]; Singapore (Malay), 0.46% [27]; Singapore (Chinese), 2.1%; Singapore (Malay), 4.5%; and Singapore (Indian), 9.7% [28]; Australia (Blue Mountains), 2.3% [29]; Australia (Victoria), 0.98% [30]; Australia (Central Australia), 16.3% [31]; Finland, 22.1% [32]; France, 5.5% [33]; Greece (Epirus), 24.3% [34]; Greece (Crete), 16.1% [35]; Greenland (Eskimos), 4.5% [36]; Iceland, 10.7% [37]; Belgrade, 17.5% [38]; Jordan, 9.1% [39]; Northern Jordan, 10.3% [40]; Iran, 13.1% [41]; Pakistan, 6.45% [42]; Saudi Arabia, 3.5% [43]; Nigeria, 2.7% [44]; Norway, 16.9% [45]; Urban South Africa, 1.4% (white population) and

20% (black population) [46]; Turkey, 7.2% [47] and 10.1% [48]; USA (Framingham), 1.8% [49]; and USA (Southeastern), 3.2% [50]. Overall, these reports suggest that genetic and environmental factors might have a major influence on the phenotypic expression of XFS.

31.3 Etiology and Proposed Pathophysiological Mechanism of Exfoliation Syndrome

The foremost pathological mechanism involved in exfoliation syndrome is the deposition of the flaky grayish-white amyloid-like material on the ocular tissues such as lens, pupillary borders, iris, trabecular meshwork, ciliary body, cornea, and lamina cribrosa toward the anterior segment of the eye that bathed by aqueous humor [3, 5]. The gradual deposition of the exfoliation material (XFM) impedes the aqueous humor flow by blocking the sieve natured layers of trabecular meshwork, Schlemm's canal, ciliary muscles, and uveal meshwork that drains out the aqueous humor through both conventional and nonconventional pathways [4, 51, 52]. This blocked or misdirected aqueous humor outflow may lead to increased intraocular pressure (IOP) of the eye, which subsequently ends with irreversible blindness leading to a clinical condition known as exfoliation glaucoma.

To understand the nature of the exfoliation material, three different theories [4] were proposed by using biochemical and immunohistochemical approaches, including (1) amyloid theory, even though XFM showed positive labelling with a crude anti-amyloid A antiserum [53] and few observed XFS cases associated with primary amyloidosis [54] but lacked any conclusive evidence. (2) Basement membrane theory was proposed due to the fact that production of XFM may disrupt the basement membrane metabolism which was also supported by frequent association of XFM and defective basement membrane of various cell types [55]. (3) Elastic microfibril theory was proposed based on the frequent structural association of the exfoliation fibers with components of the elastic system and on ultrastructural indications from the developing XFM by degenerating the elastic microfibrils [56]. Currently, the various elements representing as the precursor molecule of the dysregulated cellular mechanism involved in XFS are poorly understood.

31.4 Pathology and Clinical Features of Exfoliation Syndrome

Busacca et al. made the first histological observation in XFM which suggested that the flaky materials arose from the aqueous humor and may not be from lens capsules [57]. In contrast, electron microscopic analysis clearly suggested the involvement of the lens capsule in XFS by the presence of XFM upon the lens capsule, anterior equatorial regions, and inner half of the lens known as central disk but not in lens epithelium [58]. Other electron microscopic analysis showed the XFM deposition on the posterior surface of the iris and ciliary body along with their internal limiting membranes. XFM deposition was also observed deeply into the space between the epithelial cells of the iris, whereas their epithelial cells were normal with intact nuclei and cytoplasm. In the iris, unusual pigmented granules were noticed outside the epithelial cells, while few cells got ruptured, and those ruptured cells contained the filaments of the XFM, which were also observed in stroma and surrounding blood vessels [59]. Lectin staining with XFS lens capsule, zonules, and iris suggested the complex carbohydrate composition in XFM with O-linked sialomucin-type and N-linked oligosaccharide chains [60]. Electron microscopic immunohistochemistry observations revealed that XFM material from lenses, trabecular meshwork, bulbar conjunctivas, and lid skin almost had similar ultrastructure and identical immunoreactivity to the antibodies including vitronectin, fibronectin, laminin, and elastin suggesting XFS to be a systemic disorder [61].

The preoperative corneal endothelial cell density was reduced in XFS patients compared with age-matched controls, whereas the cataract sur-

gery in XFS patient also showed a similar induced endothelial cell changes without increase in endothelial cell loss during postoperative period [62]. In the early 2000s, Inoue et al. also reported the significant reduction of corneal endothelial cell density and thinner central cornea in XFS eyes than in non-XFS eyes [63]. The corneal subbasal nerve plexus variables, endothelial cell densities, and mean anterior and posterior stromal keratocyte were significantly lower in XFS subjects, while their basal epithelial cell density was normal [64].

The other clinical manifestations related to XFS are (a) iris depigmentation which leads to pupillary transillumination defects, (b) mild hyperpigmentation of the trabecular meshwork, and (c) zonular dehiscence which causes lens subluxation and loss of the zonular support which makes cataract surgeries more challenging with vitreous loss and lens dislocation [65].

31.5 Genetic Inheritance Pattern of Exfoliation Syndrome

Being a late-onset disorder, determining the inheritance pattern is a challenging task as genetic analysis with more than two generations is barely possible due to mortality. Despite of these complications, studies with human leukocyte antigens (HLA) in XFS support the genetic predisposition for the development of XFS. A study from Irish subjects showed the association of the 14 HLA antigens (HLA-Al, A33, B8, B47, B51, B53, B57, B62, DR3, DR12, and DR13) with exfoliation syndrome [66]. A study from Swedish XFS patients reported a significant association with HLA Bw35 compared to primary open-angle glaucoma (POAG) patients [67]. In contrast, Norwegian patients did not show association with HLA-A and HLA-B compared to POAG subjects [68]. XFS was known to be a familial condition, and the transmission to the second generation was through an affected mother in Icelandic families [69]. XFS was observed to be transmitted as a late-onset autosomal dominant trait without a maternal transmission in three Gozo families [70]. Genome-wide scan of XFS in Finnish family had demonstrated an autosomal dominant mode of inheritance with incomplete penetrance [71]. In a population-based Finnish Twin Cohort Study for determining the inheritance of XFS, 34 XFS patients were analyzed, but the heritability of XFS was not possible to assess by the classical twin method due to smaller number of twin pairs [72]. XFS perhaps is a mitochondrial disorder because of the maternal transmission tendency, 99.9% of the mitochondria inherited from the mother [73]. Reduced mitochondrial numbers was revealed in the iris sphincter muscles of XFS patients through electron microscopic analysis [74]. But it demands further studies to provide definitive proof for mitochondrial inheritance.

31.6 Clinical and Systemic Complications Associated with Exfoliation Syndrome

The subjects with XFS have been suggested to be strongly susceptible to cardiovascular risk [75] and cerebrovascular complications [76], and it had always been a matter of controversy. XFS patients are suggested to be screened for coronary artery disease (CAD), irrespective of the age and sex [77]. A meta-analysis study has elucidated that XFS has been associated with an increased risk of vascular disease [78]. Although, additional data should be acquired for the key correlations, an increased incidence of cardiovascular disorders in XFS patients and several other common features in the pathogenesis suggests that XFS may be an independent risk factor for cardiovascular disease or it may occur as a part of systemic disorders with cardiovascular implications [79]. It is difficult to obtain the accurate causality information about XFS, glaucoma, and CAD, which of the three occurred first [80]. Recently, a meta-analysis by Siordia et al. has observed the association between XFS and ischemic heart disease, while there was no correlation found with myocardial infarction, chronic ischemic heart disease, angina, and hypertension with XFS [81]. However, other studies did not show a clear association between XFS and ischemic heart disease, arterial

hypertension and diabetes mellitus [82, 83] and aortic aneurysm, and peripheral artery disease [84]. A study has reported that the prevalence of XFS in diabetes patients was shown to be less than nondiabetic patients [85]. Further, studies also showed that XFS or XFG (exfoliation glaucoma) is not correlated to mortality when either of them represented with cardiovascular and cerebrovascular complications [86, 87]. Overall, reports are still contradictory regarding the significant correlation with XFS and systemic disorders. In our study, the prevalence of hypertension, coronary artery heart disease, and diabetes mellitus was statistically higher in XFS patients when compared to the control group (Table 31.1). This significant difference in the prevalence of the systemic complications suggests the strong association with XFS. However, further studies are necessary to explore and understand the underlying mechanism.

31.7 Genetic and Nongenetic Factors of Exfoliation Syndrome

Both genetic and nongenetic factors play a role in the development of XFS. The genetic factors include the single nucleotide polymorphisms (SNPs) in the candidate genes such as lysyl oxidase-like 1 (*LOXL1*), LOXL1 antisense RNA (*LOXL1-AS1*), calcium voltage-gated channel subunit alpha1 A (*CACNA1A*), and contactin-associated protein-like 2 (*CNTNAP2*). Genes of cellular pathways implicated in XFS are homocysteine metabolism genes, adenosine receptor A3, matrix metalloproteinases (*MMPs*), glutathione S-transferase (*GST*), clusterin (*CLU*), and tumor necrosis factor *(TNF)-α*. The nongenetic factors include sun exposure [88, 89], geographical history [90], consumption of caffeine, and low folate intake [91].

31.7.1 Lysyl Oxidase–Like 1 (*LOXL1*)

Thorleifsson et al. performed the first genome-wide association study (GWAS) with 274 XFG cases/14,672 controls and identified three major single nucleotide polymorphisms (SNPs) in *LOXL1* gene on chromosome 15, which was strongly associated with XFS and XFG [92]. The two coding SNPs rs1048661 (G/T; Arg141Leu) and rs3825942 (A/G; Gly153Asp) were present in the exon 1 of the *LOXL1*, while the other SNP rs2165241 (C/T) was located near the intron 1 of *LOXL1*. These two coding variants were observed in 98% of the XFS cases but were also observed

Table 31.1 Systemic complications associated with XFS patients and cataract controls (CC)

Systemic complications	XFS	CC	*P*-value	Odds ratio (95% CI)
Electrocardiogram	*n (%)*	*n (%)*	0.145	
Normal ECG	691(86.9)	378(89.8)		1.32 (0.91–1.93)
Abnormal ECG	104(13.1)	43(10.2)		
Total	795(100.0)	421(100.0)		
Hypertension	*n (%)*	*n (%)*	0.013	
HT	215(25.2)	84(19.1)		1.43 (1.08–1.90)
Non-HT	637(74.8)	357(80.9)		
Total	852(100.0)	441(100.0)		
CAHD*	n (%)	n (%)	0.008	
CAHD	42(4.9)	8(1.8)		2.81 (1.31–6.04)
Non-CAHD	809(95.1)	433(98.2)		
Total	851(100.0)	441(100.0)		
Diabetes mellitus	n (%)	n (%)	0.033	
DM	158(18.5)	61(13.8)		1.42 (1.03–1.95)
Non-DM	694(81.5)	380(86.2)		
Total	852(100.0)	441(100.0)		

XFS exfoliation syndrome, *CC* cataract control, **CAHD* coronary artery heart disease

in 85% of the unaffected individuals. The *LOXL1* is a member of the lysyl oxidase family of proteins with five members LOX, LOXL1, LOXL2, LOXL3, and LOXL4. This *LOXL1* is a copper-dependent monoamine that is actively involved in the covalent cross-linking of the elastin and collagen polymers during extracellular matrix formation and remodeling [93]. LOXL1 knockout mice showed a distinctive phenotype such as reduced elastic fiber formation, impaired connective tissue repair, pelvic organ prolapses, skin laxity, enlarged lung airspaces, impaired blood-aqueous barrier, and lens abnormalities with cataract formation [94–98].

The association of the two coding variants rs1048661 (Arg141Leu) and rs3825942 (Gly153Asp) was globally replicated in all the populations worldwide [99]. The risk allele for these coding SNPs showed a reversal risk allelic association in one or more populations [100]. The variant rs1048661 (Arg141) showed decreased risk in Asian cohorts, while the increased risk was observed in all other populations. Likewise, the variant rs3825942 (Gly153) showed decreased risk in South Africans, whereas increased risk was seen in all other populations studied. The functional role of these variants in the pathogenesis of the disease is still obscure, but these variants are expected to be in linkage disequilibrium with any unknown functional variant, which should be answered by further genetic analysis.

Recently, epigenetic regulation of gene expression in the *LOXL1* locus has been explored in Uygur population which identified hypermethylation of CpG islands in the *LOXL1* promoter region in anterior lens capsule specimens from XFS patients compared to controls. *LOXL1* mRNA levels were reduced in the XFS lens capsules, suggesting that hypermethylation down-regulates gene expression at this locus [101]. Additionally, immunohistochemical analysis demonstrated the significant expression of LOXL1 and fibrillin-1 (FBN-1) toward outer surface of the lens capsule. Significant expression of LOXL1 in lens epithelial cell nuclei was observed, whereas the cytoplasmic expression of LOXL1 was lower in XFS patients [102].

31.7.2 LOXL1 Antisense RNA (*LOXL1–AS1*)

Hauser et al. showed many risk variants at *LOXL1* exon 1/intron 1 which are strongly associated with XFS in four different ethnic populations; some were reversed in one or more populations. The intron 1 region comprises of a promoter for the long noncoding RNA (lncRNA) *LOXL1-AS1*, which is on the opposite strand of *LOXL1*. The expression of *LOXL1-AS1* was affected due to XFS-related cell stressors, including oxidative and mechanical stress. Remarkably, a haplotype of risk alleles for three intronic SNPs is shown to be reducing the promoter activity of *LOXL1-AS1* by 43% in lens epithelial [103]. As lncRNAs transcription is sufficient to regulate gene expression either positively or negatively at both neighboring and distant loci [104], now the altered *LOXL1-AS1* expression may affect expression of multiple other XFS-related genes located throughout the genome. Thus, it suggests an interesting possibility that dysregulated expression of the *LOXL1-AS1* lncRNA may play a role in the pathogenesis of XFS and XFG.

31.7.3 Calcium Voltage–Gated Channel Subunit Alpha1 A (*CACNA1A*)

A multicentered GWAS reported the association between the *CACNA1A* locus and XFS susceptibility with 7000 XFS cases and 20,000 controls from 17 countries [105]. The associated variant rs4926244 that lies in an intronic region near the 3′ end of the *CACNA1A* was first observed in Japanese population then replicated as multicentered GWAS analysis. The risk allele for rs4926244 was shown to be consistent across all major populations, with an allele frequency ranges between 10 and 40%. The in silico analysis suggests that the risk allele for rs4926244 is associated with the reduced CACNA1A mRNA expression levels in peripheral blood cells [105]. In accordance with this, another study has demonstrated a moderate mRNA expression of *CACNA1A* in the trabecular meshwork, lens cap-

sule, and retina, as well as a low expression in the cornea, iris, ciliary body, and choroid by comparing 20 XFS eyes with 20 control eye; however, no significant difference could be established [106].

CACNA1A encodes the α1A subunit of the type P/Q voltage-dependent calcium channel on chromosome 19. Calcium channels are said to be responsible for the transport of calcium ions across cell membranes and play a vital role in a cell's ability to generate and transmit electrical signals. An electron microscopy studies on human XFS eyes showed the presence of high calcium concentrations in direct association with aggregating XFS fibrils [107]. Thus, it can be postulated that the dysregulated function of a calcium channel could lead to alterations in calcium concentrations, which may result in the formation of XFS aggregates [105].

Recently, the expanded GWAS on XFS from 24 countries followed by the replication in 18 countries identified the genetic association of a rare protective allele at *LOXL1* (rs201011613: A/T; p.Phe407) and 5 new genome-wide significant susceptibility loci (i) rs7329408 (A/G): *POMP*:13q12, (ii) rs11827818 (G/A): TMEM136: 11q23.3, (iii) rs3130283 (A/C): AGPAT1: 6p21, (iv) rs12490863 (A/G): RBMS3: 3p24, and (v) rs10072088 (G/A): SEMA6A:5q23 as an additional clarification for the genetic basis of XFS [108].

31.7.4 Other Genetic Associations of Exfoliation Syndrome

The candidate gene studies have identified some of the other positive and negative genetic associations between XFS risk and *CNTNAP2* [109–111], *CLU* [112–115], GST [116], TNF-α [115, 117], and baculoviral IAP repeat-containing 6 (BIRC6) [118]. Nevertheless, these genetic associations have not been replicated in a larger, multiethnic GWAS for XFS [105, 108], which reveals that these associations may be weak or limited to the specific ethnic population.

Additionally, Izzotti et al. have reported the mitochondrial DNA deletion in XFG cases and suggested that mitochondrial failure might play an essential role in the functional decay of trabecular meshwork [119], while another study did not show any association with the mitochondrial DNA [120]. Recently, Want et al. reported that the Tenon fibroblast cells of XFS display features associated with autophagy and mitochondrial dysfunction [121].

31.8 Summary

Molecular genetic approaches stand to be a powerful tool to unravel the genetic basis of any disease. Candidate gene studies are necessary to unlock the heritable locks involved in the pathogenesis of the complex genetic disorders including exfoliation syndrome. Numerous research efforts like multicentered genome-wide association studies have been carried out to understand the complicated mechanism involved in the exfoliation syndrome. The future research should be focused to underpin the major functional component which is acting at the background of the all well-studied high-risk candidate genes associated with exfoliation syndrome.

Compliance with Ethical Requirements Prakadeeswari Gopalakrishnan, Aravind Haripriya, Banushree Ratukondla, and Periasamy Sundaresan declare that they have no conflict of interest. No human or animal studies were performed by the authors for this article.

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Proteomics of Neurodegenerative Disorders of the Eye

32

Kim Ramasamy, Krishnadas Ramasamy, Dharmalingam Kuppamuthu, and Jeya Maheshwari Jayapal

Abstract

Neurodegenerative eye diseases refer to those ocular conditions where the retina is affected and include pathologies such as diabetic retinopathy, age-related macular degeneration, and glaucoma. All these three diseases are the leading causes of irreversible blindness. Although they affect different parts of the retina, the death of retinal cells that is responsible for vision loss is the common unifying factor of the three conditions. These diseases are generally multifactorial, relatively asymptomatic, and progressive. Proteomics of the tissues and fluids of the posterior compartment of the eye has advanced our understanding on the pathological mechanisms underlying all three conditions. These studies have also contributed to the identification of candidate biomarkers that upon validation would be helpful in the early diagnosis of these diseases.

Keywords

Neurodegeneration · Age-related macular degeneration · Diabetic retinopathy · Glaucoma · Proteomics

32.1 Introduction

Diabetic retinopathy, age-related macular degeneration, and glaucoma are the three major degenerative diseases of the retina that causes irreversible blindness. All the three diseases have been included in the list of priority eye diseases of the World Health Organization as they have emerged as potential threats, particularly for the middle-aged and the ageing population of many middle-income and industrialized countries.

Age-related macular degeneration (AMD) ranks third among the global causes of visual impairment and is the primary cause of legal blindness in many countries. It is a chronic and progressive disease of the central retina of the elderly population. There are two subgroups of AMD, atrophic (dry form) and exudative (wet form). In most patients, the AMD onsets as the dry form, which can advance either to the late dry AMD (geographic atrophy) or linked to choroidal neovascularization (CNV) resulting in wet AMD. Geographic atrophy leads to progressive vision loss, with a decrease in dark-adapted function and a significant drop in acuity. On the other hand, neovascular AMD causes sudden and severe vision loss.

K. Ramasamy
Retina-Vitreous Services, Aravind Eye Hospital, Madurai, India

K. Ramasamy
Glaucoma Services, Aravind Eye Hospital, Madurai, India

D. Kuppamuthu · J. M. Jayapal (✉)
Department of Proteomics, Aravind Medical Research Foundation, Madurai, India
e-mail: jeya@aravind.org

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The increase in diabetes among many population groups has caused diabetic retinopathy (DR) to be added to the WHO's priority eye diseases. DR is a microvascular complication that develops in both type 1 and type 2 diabetics. Beyond 20 years of diabetes, nearly all type 1 and 50–80% of type 2 diabetic patients have some form of DR [34]. The early stage of DR is non-proliferative (NPDR), which progresses from mild to moderate to severe form. A subset of NPDR patients progress further to the advanced stage characterized by neovascularization called proliferative diabetic retinopathy (PDR). At any stage of DR, patients may also develop macular edema, which is exudation and accumulation of fluids in the macular region. Both DME and PDR are associated with poor visual outcomes.

Glaucoma has remained a public health concern for long due to difficulties in its early diagnosis combined with a need for lifelong treatment. Glaucoma is asymptomatic in the early stages, and the disease advances with substantial neural damage. When symptoms do occur, there is already a significant reduction in the visual field that greatly affects the quality of life. Glaucomas can be classified as open-angle glaucoma or angle-closure glaucoma. Both these types of glaucomas can be primary diseases with characteristic optic neuropathy and a normal or elevated IOP with no other specific pathological cause. Secondary glaucoma refers to an increased IOP with an identifiable pathological cause, such as trauma, certain medications like corticosteroids, inflammation, tumor, pigment dispersion, or pseudoexfoliation. As primary open-angle glaucoma (POAG) and primary angle-closure glaucoma (PACG) account for the majority of glaucomas, these two types will be discussed here.

32.2 Epidemiology: Global and Asian

The prevalence of the three ocular diseases in the global scenario as well as with specific reference to the Asian population is shown in Table 32.1.

Table 32.1 Comparison of the prevalence of AMD, DR, and glaucoma worldwide and in Asian population

	Prevalence (%)	
	Global	Asian
Age-related macular degeneration [63]		
Early AMD	8.01	6.8
Late AMD	0.37	0.37
Any AMD	8.69	7.38
Glaucoma [6]		
POAG	3.05	2.31
PACG	0.5	0.73
Any glaucoma	3.54	3.54
Diabetic Retinopathy [38, 54]		
Among type 1 diabetics	77.3	5.3–15.1
Among type 2 diabetics	25.2	12.1–23.0

32.3 Etiology

The exact cause(s) for any of these three neurodegenerative diseases have not been clearly defined, but many studies over the years have implied various factors as probable causes. AMD, DR, and glaucoma are all multifactorial diseases, and many factors (such as genetic, environmental) do have a role in the onset of the disease.

The hallmark of AMD is the appearance of drusen, the extracellular deposits that accumulate between the basement membrane of the retinal pigment epithelium (RPE) and Bruch's membrane. Basal linear deposits between the RPE basement membrane and the inner collagenous zone of Bruch's membrane is a specific marker for early AMD [51]. These deposits lead to progressive defects in macular functions. Accumulation of RPE lipofuscin that appears as diffuse and irregular patches of autofluorescence is the earliest sign of AMD. Confluent areas of RPE cell death with a concomitant atrophy of the overlying photoreceptors are characteristic of late stage of dry AMD, also called geographic atrophy. In wet AMD, hypoxic-driven VEGF secretion induces choroidal neovascularization that can lead to subretinal or sub-RPE hemorrhage.

Important risk factors in the development of DR are long duration of diabetes, poorly controlled hyperglycemia, and hypertension. Chronic hyperglycemia induces changes in both the

neural retina and retinal vasculature. Apoptosis of RGCs and activation of glial cells result in a local inflammatory response in the neural retina. Concomitantly, changes in the vascular endothelium result in increased leukostasis, an early event that leads to occlusions in blood vessels. Many factors, particularly the dysregulation of insulin signaling and formation of advanced glycation end products, have a role in the initiation of changes in the retina.

Glaucoma has been conventionally thought to be due to high intraocular pressure (IOP). Although many individuals with high IOP may not develop glaucoma, increase in IOP is considered as a risk factor. In open-angle glaucoma patients, there is an increase in resistance to aqueous humor outflow through the trabecular meshwork (TM), while in angle-closure patients, the access to the drainage through TM is obstructed by the iris. PACG is a more aggressive form of glaucoma and is the dominant form among Asian population [17]. Disorders of the iris, the lens, and retrolenticular structures can cause PACG. Non-pupil block mechanisms have been shown to be responsible for angle closure in Asian patients [50].

32.4 Pathology

A number of basic and clinical research studies in the last few decades have identified multiple pathological events underlying the neurodegenerative eye diseases. Specific hallmarks characterize the stages of the disease as they progress and are discussed here briefly.

AMD affects primarily the RPE, photoreceptors, and Bruch's membrane. In the nonexudative form, the early stage is characterized by the appearance of basal laminar deposits (BLamD) that increase with AMD progression, and the late, amorphous form of BLamD correlates with the severity of RPE degeneration [51]. Analysis of the drusen components revealed the presence of a large number of molecular constituents including proteins such as immunoglobulins, complement components, and other proteins implying the role of immunological and inflammatory process in AMD pathology. This is further supported by the presence of macrophages in areas where the Bruch's membrane is thin or breached [30]. The growth of choroidal blood vessels through the Bruch's membrane into the RPE or subretinal space can lead to hemorrhage resulting in severe vision loss.

The early stage of DR is characterized by retinal vascular changes such as thickening of the basement membrane and loss of pericytes. Hyperglycemia-induced changes in the retinal cells include activation of glial cells, which along with RPE have important roles in early microvascular impairment. Progression of NPDR is characterized by severe endothelial damage, which together with vasoconstriction and capillary occlusion results in pathological angiogenesis. In the proliferative stage, severe hypoxia leads to an increase in the pro-angiogenic factors resulting in neovascularization. These new vessels can grow into the vitreous body, and since they are fragile, they have a tendency to rupture and leak blood into the vitreous humor.

Glaucoma is a heterogeneous group of diseases, and the pathophysiology of glaucoma is believed to be multifactorial. Many theories of glaucoma progression have been proposed and are reviewed by Davis et al. [8]. According to the mechanical theory, increased IOP might lead to the compression of the nerve fiber bundle causing a discontinuity in the axonal transport of neurotropic factors resulting in apoptotic death of RGCs. Yet another ischemic theory of glaucoma progression suggests that hemodynamic alterations independently or in conjugation with raised IOP result in optic nerve head damage. And, mitochondrial dysfunction and RGC axonal transport dysregulation activate a series of events culminating in RGC cell death. Loss of RGCs by apoptotic cell death leads to a decrease in the function of these cells that is considered to be responsible for loss of visual field.

32.5 Clinical Features

AMD, DR, and glaucoma are progressive diseases that are asymptomatic in the early stages. Their diagnosis is dependent on the clinical features.

Patients in the early stages of AMD clinically exhibit focal thickening of Bruch's membrane along with areas of hypo- and hyperpigmentation in the RPE. Patients who progress to geographic atrophy exhibit a sharp demarcated area of depigmentation indicative of RPE atrophy. And, those who develop neovascular AMD have RPE atrophy with subretinal or intraretinal hemorrhage. Neovascular AMD patients develop rapid vision loss, while it is slower over a period of many years in geographic atrophy.

The earliest detectable clinical features of DR are the presence of microaneurysms and dot intraretinal hemorrhages, both of which increase in number as the disease progresses along with the additional appearance of cotton wool spots. Advancement to PDR stage is clinically presented with the formation of new blood vessels that upon rupture can leak blood into the vitreous. Additionally, blood vessels can grow into the vitreous body resulting in the detachment of the retina. Both vitreous hemorrhage and retinal detachment are sight-threatening conditions in DR.

Distinct and characteristic changes appear in the optic nerve head and retinal nerve fiber due to RGC cell death and loss of optic nerve fiber. These changes can be identified during ophthalmoscopic examination of the optic nerve head. In glaucoma patients, there is a progressive deterioration of visual fields, with an initial loss in the peripheral vision that progresses in a centripetal manner until only the central vision remains.

32.6 Proteomics of Neurodegenerative Eye Diseases

32.6.1 Understanding the Pathological Mechanisms

A large number of studies in the last two decades have immensely contributed to our understanding of the molecular events underlying these diseases, and proteomic studies have a major contribution. Here, we discuss primarily how proteomic analysis have advanced our knowledge in these areas. The retina is the ocular tissue that is primarily affected in all these three diseases. Comparative analysis of the retina or vitreous humor (VH) from the patients and healthy donors allows the identification of disease-specific proteome alterations. Proteins altered in the early stages might represent the causative mechanisms, while those identified in the late stages could possibly be due to the accumulated effect or secondary consequences.

Proteome analysis of RPE, Bruch's membrane, and choroid-RPE complex has identified a number of factors involved in AMD pathology. Many studies have shown the involvement of complement cascade, inflammation, and oxidative stress in AMD [13, 44, 67]. Dysregulation of the complement system activation is an established pathological component of AMD. Genetic variations in the complement genes, namely, the complement factor H [12], the central complement component C3, and factors B and C2 [20], have been associated with the AMD susceptibility. An increase in the level of the complement pathway proteins has been reported in the AH [66], VH [36], RPE cells, and choroid-RPE complex [55] of AMD patients. Further, complement proteins are detected in the drusen deposits and Bruch's membrane of AMD patients [67]. The complement C3 and complement regulator, vitronectin, was reported to be elevated in wet AMD tissues but not in the advanced dry AMD tissues [67].

AMD affects both the macula and the periphery of the retina. Evidence for this comes from the study by Ethen and group [13] who demonstrated that 60% of the altered proteins are specific to either the macula or periphery. Inflammation-related proteins are elevated in the foveal or macular region, while bestrophin 1, rhodopsin, ras homolog family member A, and ras homolog family member C are the proteins found in high levels in the periphery.

Proteomics have been helpful in identifying the stage-specific changes in the retina during AMD progression. Yuan et al. [67] established a database of 901 proteins in macular Bruch's membrane/choroid complex of AMD and normal donors. Complement proteins, DAMPs, and

other immune response and host defense proteins were found to be increased in AMD suggesting the involvement of inflammatory processes in both initiation as well as progression of the disease. RPE cells perform a range of complex functions that are important for proper visual function. Loss of function of RPE cells are the initial events in atrophic AMD ultimately leading to death of these cells. Nordgaard and group [44] identified the changes in the RPE proteome of human donor eyes across four progressive stages of AMD. Impaired stress response and mitochondrial biogenesis were observed in the early stages of AMD. Proteins involved in apoptotic signaling pathways were altered as the disease progressed and are of significant concern as RPE cells have limited ability to renew. αA and αB crystallin levels were found to be increased indicating a cellular response to oxidative stress [13]. As four retinoid processing proteins were uniquely elevated in early- or mid-stage AMD, retinoids and lipofuscin might be presumed to play a role in AMD initiation. Proteins involved in cellular growth and proliferation, hematological disease, and cell morphology were found to be particularly altered in the early or mid-stage of the disease [67]. Late-stage changes were observed in proteins that regulate retinoic acid and regeneration of the rhodopsin chromophore. Distinct sets of proteins are altered in advanced dry AMD and neovascular AMD implying the differences in the mechanisms of progression. Galectin-3, the advanced glycation end product receptor 3, is the most significantly elevated in more than 80% of advanced dry AMD samples, while neutrophil α-defensins 1–3 are the most abundant in neovascular AMD. Both forms of advanced AMD samples showed increases in metalloproteinase inhibitor 3 and S100-A9 level. Cellular compromise, drug metabolism, and molecular transport proteins decreased in geographic atrophy, whereas cell cycle, tissue development, and cellular development-related proteins were at lower levels in neovascular AMD [67].

In diabetic retinopathy, changes in the neural retina occur even before the onset of clinical symptoms in the retinal vasculature. Examining the retinal proteome in the diabetic and early DR stages has been valuable to understand the early changes leading to neurodegeneration of the retina. A 2D-based proteomic study on diabetic human donor RPE identified the molecular changes associated with diabetes prior to the onset of DR [9]. Majority of the proteins altered in diabetic RPE were involved in metabolism, chaperone function, protein degradation, synthesis and transport, oxidoreductases, cytoskeletal structure, and retinoid metabolism. Many of these proteins have been reported to be altered during diabetes in non-ocular tissues, and this is the first report on their alteration in the retina. Proteomics of the human retina from DR donor eyes is quite limited. Most of the studies on retinal proteome changes have been carried out using animal models. Rat is a widely accepted model as they exhibit histological changes in the early stages of diabetes similar to that observed in humans. Quin and group [48] established a 2D proteome map of the rat retina to study the proteome-wide changes induced by early diabetic retinopathy. Proteins were differentially expressed as well as posttranslationally modified. Some proteins such as HSPs 70.1A and 8, and platelet-activating factor, were uniquely expressed in diabetic retina, while beta-catenin, phosducin, aldehyde reductase, succinyl-CoA ligase, and dihydropyrimidase-related protein were differentially regulated. Ya-Dong and group [64] also published a similar proteome-wide study on rat retina. This study identified numerous proteins including αA-crystallin, glyceraldehyde-3-phosphate dehydrogenase, and glutamine synthetase to be differentially expressed in the rat neural retina suggesting that diabetes induces complex changes in the retina. Quin's group induced DM in rats using streptozotocin that creates an insulin deficiency, and thus, the retinal proteome reflects the changes as in type 1 diabetes. On the other hand, Wang's group generated a type 2 diabetes model by feeding rats with high-fat diet followed by a low-dose injection of streptozotocin that creates only insulin resistance, and not deficiency. Altered expression of crystallins was also reported in diabetic rat retina by Fort et al. [14]. They showed increased expression of the isoforms of α-, β-,

and γ-crystallin, and their 2D-DIGE approach was helpful in identifying that these crystallins undergo multiple posttranslational modifications during diabetes. The authors also demonstrated that γ-crystallins and β-crystallins were upregulated in the ganglion cells and inner retinal neurons, respectively. As the upregulation of these crystallins coincided with the onset of DR symptoms, these proteins might have a role in vascular remodeling in diabetes. A little later, VanGuilder et al. [61] used a multimodal proteomic approach to achieve an in-depth coverage of the retinal proteome that also validated the protein alterations reported earlier. However, the authors did not find any difference in VEGF expression, which is contradictory to the already reported increase in VEGF expression. Ly and group [43] studied retinal membrane proteome of 10-week-old diabetic db/db mice treated with antihyperglycemic drug. Proteome alterations include a decreased expression of proteins related to synaptic transmission (e.g., vesicular glutamate transporter 1) and cell signaling in diabetic mice. This study also shows that antihyperglycemic agents can only partially ameliorate diabetes-induced changes in the membrane-associated signaling proteins.

Additional insights on the pathogenic mechanisms in DR come from the proteome analysis of VH, AH, and serum/plasma. Using in-depth profiling methods, the changes in the vitreous proteome have been analyzed in both NPDR and PDR stages. Proteins involved in complement, coagulation, and kallikrein-kinin system have been consistently found to be altered across many studies [2, 16, 40]. In addition, apolipoproteins, immunoglobulins, and cellular adhesion molecules are also altered [40]. Many studies demonstrate the involvement of factors other than VEGF in vascular changes. Dyer et al. [11] showed that carbonic anhydrase mediates an increase in vascular permeability as observed in DME and PDR. Beta-2 integrin pathway is yet another factor leading to VEGF-induced changes during DR [27]. Garcia-Ramirez [19] studies on the retinal proteome detected lower mRNA and protein levels of interphotoreceptor retinoid-binding protein (IRBP) with a concomitant increase in the GFAP levels (an indicator of cell damage), implying a role for IRBP in neurodegeneration. Two studies specifically examined changes in the DR vitreous with special reference to diabetic macular edema, as this is a risk factor for vision loss in DR patients. These studies show increase in hemopexin, PEDF, ApoA4, ApoH, Trip-II, PRBP, and VDBP and decrease in clusterin, transthyretin, and crystallin-S, and these changes might be DME specific [23, 45].

Proteome analysis of the retina from glaucomatous human donor eyes as well as animal models provides an insight into glaucoma-induced alterations. Tezel and group published a series of finding, all based on the proteomics of glaucomatous retina. Initially, through gel-based analysis, they identified the proteins that were altered as well as posttranslationally modified in glaucomatous rat retina [57, 65]. These studies demonstrated the involvement of 14-3-3 proteins in cellular signaling in glaucomatous neurodegeneration. Later, through a quantitative gel-free 2D-LC-MS/MS, Tezel and group [58] identified a large number of proteins that were differentially regulated in both human and rat retina during glaucoma. A twofold upregulation of hemoglobin expression was detected in the retina as well as the optic nerve head macroglia. Additional in vitro experiments indicated that glial expression of Hb was induced by hypoxia through EPO signaling. The same group also suggested that the complement system [59] and TLR signaling [42] are activated in the human retina during glaucoma. Activation of complement through classical and lectin pathway was also accompanied by a decrease in the levels of complement factor H [59]. Stowell and group [56] used nonhuman primate model of glaucoma, and using a label-free quantitative MS methods showed that cytoskeletal remodeling may play a role in the early retinal response to chronic IOP elevation. Tezel and group [59] compared the human retinal proteome of ocular hypertensive donor with that of glaucomatous and normal donor to understand how high IOP leads to neurodegeneration [59]. This study identified an initial period of intrinsic stress and defense response during ocular hypertension, and these responses along with an ongoing cell death

process characterized glaucoma. A recent study on the human retinal proteome of glaucoma patients suggest that an impairment of energy metabolism and stress response occurs during the process of retinal neurodegeneration [15]. These changes were also reflected in the aqueous humor of glaucoma patients [25]. Bhattacharya and group [3] examined the optic nerve to understand the molecular pathology of POAG. Through proteomic approach, they identified PAD2 protein to be present exclusively in glaucomatous optic nerve and also provide evidence for the translational modulation of PAD2 expression suggesting that this enzyme might be involved in optic nerve damage in POAG. Some studies have examined the proteome of cultured trabecular meshwork (TM) to identify changes in response to treatment with dexamethasone [5] or TGF-β2 [69]. These studies suggest that both treatments induce changes in the ECM and secreted proteins in TM, similar to changes observed in glaucoma. Other studies have implicated cochlin, a deafness disorder protein [4], and copine1, a calcium-dependent membrane-binding protein [68] to be increased in glaucomatous TM. Grus et al. [22] identified transthyretin to be present at high levels in the AH of glaucomatous patients and proposed that this protein might play a role in the onset of glaucoma through the formation of amyloid deposits. Duan and group [10] showed significant alteration in AH proteome of POAG patients, and a study by Kaeslin and group [28] suggested the proteome changes in AH reflect an imbalanced metabolism, lack of ROS detoxification, and low-grade and chronic inflammatory processes that occur during glaucoma. Proteomic studies have mostly been carried out for POAG, and surprisingly, proteomics of PACG is a completely unexplored area.

32.6.2 Disease–Specific Candidate Protein Markers

Quantitative proteomic studies identify proteins that are significantly altered in a pathological condition. These proteins may or may not be a part of specific pathways but are involved in the disease pathology. Hence, the differential presence or alteration in the level of proteins can be used as a marker. Many such markers have been identified in the retina, VH, AH, and serum/plasma for AMD, DR, and glaucoma. Those markers identified through proteome analysis are considered in this review, and some of these have already been discussed in the previous section. Additional markers are listed here.

Early diagnosis and treatment of AMD are critical for preventing AMD-related blindness. Phospholipid transfer protein (PLTP) and mannan-binding lectin serine protease (MASP-1) have been suggested as plasma biomarkers for early AMD. The discriminatory power of these biomarkers is enhanced when combined with the risk genotypes, age-related maculopathy susceptibility 2, and complement factor H genes [31]. Vinculin is another potential plasma biomarker for AMD [32]. Proteome analysis of the exosomal proteins in AH enabled the identification of molecular chaperone proteins, and proteins related to the autophagy-lysosomal pathway have been identified as potential biomarkers and therapeutic target proteins for AMD [29]. Pharmacological intervention either to target Src kinase with the aim of preventing cytoskeletal rearrangements in the retinal pigment epithelium (RPE) and neuronal retina or to help rebuild damaged IPM may provide fresh avenues of treatment for patients suffering from AMD [37].

Proteome studies on DR have identified many candidate protein markers such as apolipoproteins (ApoA-1, ApoH), complement system proteins (C3, FI, C4b, C9, CFB), coagulation cascade proteins (prothrombin, antithrombin III, factor XII, fibrinogen A), and other proteins like alpha-1 antitrypsin, peroxiredoxin, zinc-alpha glycoprotein, and angiotensinogen [2, 16, 18, 52] that were increased in the vitreous humor. On the other hand, proteins that were present at lower levels in DR vitreous include PEDF, IRBP, ITIH3, calsyntein-1, interphotoreceptor retinoid-binding protein, neuroserpin, and extracellular SOD [16, 18]. Wang and group [62] through a 2D-based MS approach reported for the first time alterations in DDAH1, tubulin α-1B chain, γ-enolase, cytosolic acyl-CoA thioester hydro-

lase, malate dehydrogenase, and phosphatidylethanolamine-binding protein 1. Many groups have examined the serum or plasma profile to identify candidate biomarkers for DR. Proteins, namely, hemoglobin, CD160 antigen, β2-GPI, AHSG, α1-AGP, and apo-A1, were elevated, while afamin, protein arginine N-methyltransferase, vitamin binding protein, gelsolin, RBP1, NUD10, and neuroglobin were detected at lower levels in the serum of DR patients [21, 39, 41]. Kim and group [33] adopted a comprehensive approach where they identified proteins to be altered in the VH of DR patients and combined it with those reported in literature. They subsequently validated these markers in NPDR patient serum and suggested 28 candidate proteins that can distinguish moderate NPDR group from DM patients without DR. Further, they proposed a more specific multi-biomarker panel for the early-stage DR comprising of apo-4, complement C7, clusterin, and inter-alpha-trypsin inhibitor heavy chain H2 [26, 33]. Two groups identified circulating antibodies to be DR specific. Sinha et al. [53] propose anti-MPO antibody as a marker for the progression from NPDR to PDR as the levels of this antibody correlated with the severity of DR. Ahn et al. [1] examined specifically for anti-retinal autoantibodies by screening the retinal proteins using human sera from DM and DR patients and found anti-aldolase antibody to be elevated in DR patients. Ting and group [60] have reviewed the biomarkers for diabetic retinopathy from all other studies and have provided a comprehensive list of vitreous and serum biomarkers.

Crabb and group [7] quantified proteomic change in aqueous humor (AH) from human POAG donors. Among the differentially expressed proteins in AH, carbonic anhydrase 1, Cu-Zn superoxide dismutase, and insulin-like growth factor-binding protein 7 were upregulated, while C-Jun-amino-terminal kinase-interacting protein 4, glutathione synthetase, synaptotagmin 5, and ten different crystallins were downregulated. Nearly 44% of the AH proteins were detected in the patient plasma suggesting that glaucoma-induced changes can be monitored in the blood. Additional proteins such as ELAM 1, apolipoprotein B and E, phospholipase C, muscle cell differentiation and function proteins such as myotrophin, and heat shock proteins (Hsp60, Hsp90) also show differential expression in the AH of POAG patients [49]. Grus et al. [22] reported transthyretin as one of the most abundant proteins in the AH of glaucomatous patients. On the other hand, autotaxin is an abundant protein in normal AH, but its lysophospholipase D activity was significantly elevated in glaucoma [24]. Pieragostino and group [46] profiled the proteome of tears of newly diagnosed and untreated glaucomatous patients (naïve) with that of patients undergoing therapy with prostanoid analogues. Proteins, namely, lipocalin-1, lysozyme C, lactotransferrin, proline-rich-protein 4, prolactin-inducible protein, zinc-alpha-2-glycoprotein, polymeric immunoglobulin receptor, cystatin S, Ig kappa chain C region, Ig alpha-2 chain C region, immunoglobulin J chain, and Ig alpha-1 chain C region, were all upregulated in naïve POAG patients. Some of these proteins have also been found to be altered in the AH of glaucomatous patients [47]. Biomarkers for POAG from different tissues (TM, optic nerve) and fluids (AH, serum/plasma) with reference to the specific functional category are discussed in detail by Knepper et al. [35].

32.7 Limitations

Proteome analysis of the retinal cells and ocular fluids has been very helpful in identifying the factors and events that contribute to the pathology of these three neurodegenerative eye diseases. Many of these studies have also identified candidate biomarkers. Upon validation in a larger dataset, these proteins will be useful in predicting the high-risk subgroups or for an early diagnosis. However, the limitation lies in the source of the tissue or fluid for assaying these markers. Many of these markers have been identified in the retina, VH, or AH of patients, and these tissue or fluids cannot be collected from normal individuals for ethical reasons. Hence, it is important to identify markers for these retinal diseases in sources such as tears or blood that is easily acces-

sible as well as those that are disease specific. Yet another limitation lies in the amount of tissues/fluids available for proteomic analysis, and in many cases, the starting material is insufficient for multiple analysis. Proteomic technology has advanced exponentially, and the newer platforms generate a huge amount of data. Also, a parallel development in the analysis software is lacking, and as a result only a small percentage of the mass spectrometry data is translated to information.

32.8 Summary

Advancements in proteomic technology have resulted in the availability of high-throughput and sensitive methods to carry out large-scale profiling as well as quantitation of proteins in clinical samples. Studies using both gel- and non-gel-based quantitative methodologies have resulted in the in-depth profiling of the retina, RPE, optic nerve, and vitreous and aqueous humor from normal individuals and patients. We now have a database of proteins that are altered in these tissues/fluid across the three different pathologies. Many studies have identified a number of proteins and pathways that play a role in the initiation and progression of the three neurodegenerative eye diseases. These studies have made a significant impact on our understanding of the disease and have resulted in identification of many biomarkers and therapeutic targets.

Compliance with Ethical Requirements R. Kim, R. Krishnadas, K. Dharmalingam, and J. Jeya Maheshwari declare that they have no conflict of interest.

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Genomic Approaches to Eye Diseases: An Asian Perspective

33

Bharanidharan Devarajan, Ayyasamy Vanniarajan, and Periasamy Sundaresan

Abstract

Recent advances in genomic technologies, particularly next-generation sequencing (NGS) methods, have brought a paradigm shift in discovering eye disease-associated genetic variants from linkage and genome-wide association studies to NGS-based genome/exome studies. Whole-genome sequencing (WGS) remains prohibitively expensive for most applications and requires concurrent development of bioinformatic approaches to expeditiously analyze the large data sets; whole-exome sequencing (WES) is now made as a viable approach to uncover unknown etiology with a limited number of probands with eye disease. WES focuses on only the protein-coding sequence of human genome, has become a powerful tool with many advantages in the research setting, and moreover is now being implemented into the clinical diagnostic arena. Here, we review the current literature on technical approaches and to provide recommendations for bioinformatic analysis focusing WES and WGS methods. We highlight its successful applications for identifying causative variants in various eye diseases with emphasis on Asian data and discuss its implementation in the clinical settings.

Keywords

Genome sequencing · Exome sequencing · Variant filtering · Eye diseases · Molecular diagnosis · Asian population

33.1 Introduction

The latest WHO figures state about 180 million people are visually impaired; 22 million are blind in Asia alone. Over 526 ocular clinical phenotypes have been shown with inheritance in Online Mendelian Inheritance in Man database (https://omim.org/), which may lead to low vision and blindness. The inheritance of eye diseases, attributed to the deleterious genetic alterations, relies on a large population or aggregation of a disease in the generations of family. The identification of deleterious genetic alterations contributing to Mendelian and complex eye diseases is the first step toward the development of genetic screening tests and novel therapeutic interventions. The high-throughput genome-wide association studies (GWAS) have been used extensively to identify genetic risk factors of inherited eye diseases. However, it has had several complications due to

B. Devarajan (✉)
Department of Bioinformatics, Aravind Medical Research Foundation, Madurai, India
e-mail: bharani@aravind.org

A. Vanniarajan · P. Sundaresan
Department of Molecular Genetics, Aravind Medical Research Foundation, Madurai, India

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incomplete penetrance and linkage disequilibrium, and it can find only the common risk variants [1]. The novel and rare deleterious variants, which require to account for much of the inheritance, remain uncovered in GWAS [2]. This could be circumvented by conventional Sanger DNA-sequencing method. Despite steady improvement in the Sanger method, it remains costly, time-consuming, and low throughput. The advent of next-generation sequencing (NGS) has emerged as a high-throughput genomic approach to overcome all the limitations.

Next-generation massively parallel sequencing technology enables the comprehensive genetic sequencing of all or part of the human genome, and its improvements in chemistries and workflows have paved the way into the clinical diagnostic arena. Its efficiency enables the availability of high-speed and low-cost genomic sequencing of multiple samples. The whole-genome sequencing (WGS), i.e., sequencing the entire genome, and whole-exome sequencing (WES), i.e., sequencing the protein-coding regions alone, advance the discovery of novel variant/gene and therapeutic interventions. WES has been the successful cost-effective tool for the discovery of casual variants associated with simple Mendelian as well as complex inheritance patterns. WES has become available as a diagnostic test performed in Clinical Laboratory Improvement Amendments-certified and College of American Pathologists-accredited clinical laboratories [3, 4]. WGS is now technically feasible and cost-effective; however, it is still expensive as the cost of data analysis and storage has been much higher than initially expected. Nevertheless, recent developments in NGS platforms with faster turnaround times are accelerating the NGS implementation into the clinical domain. Yet, NGS labs have been challenged with new chemistries and instrumentation as well as complexity in the data analysis.

In this review, first, the current technical aspects of exome and genome sequencing, followed by the bioinformatic considerations and challenges, are discussed. Subsequently, the applications of NGS technology to analyze whole exomes and genomes for the causative variants discovery in eye diseases are highlighted and concluding its use and challenges in the clinical settings.

33.2 Whole–Genome and Whole–Exome Sequencing–Technical Design

Although the NGS platforms employ different sequencing approaches, they share a common feature that is massively parallel sequencing of clonally amplified or single DNA molecules that are spatially separated in a flow cell. It generates hundreds of megabases to gigabases of DNA sequence from each run based on the platforms. In recent past, it has improved immensely since their advent in 2015 that the current Illumina NovaSeq 6000 platform is capable of producing six TB of sequencing data per run. The new NGS systems called single-molecule sequencers from Pacific Biosciences [5] and Nanopore [6] can provide high read lengths and resolution of DNA modifications. Since most of published exome and genome studies have used Illumina technology, the following sections will focus on Illumina sequencing.

WES only covers 1–1.5% [7] of the human genome and operationally defined by the consensus coding sequence (CCDS) database (http://www.ncbi.nlm.nih.gov/CCDS/CcdsBrowse.cgi), yet this portion of the genome houses approximately 85% of the known disease-causing variants [8]. To achieve WES as technically possible, complete coverage of all exons of known disease-associated gene is needed. This is still a challenging process that certain regions in the genome are difficult to sequence including GC-rich regions, repeat expansions (interspersed or tandem repeat), and regions of high sequence homology (from pseudogenes or gene families) and difficult to analyze bioinformatically as well. Further, large insertions and deletions (indels), copy number variants, and other structural variants are very difficult, which can be achieved using WGS as it covers complete genome.

Basically, the routine sequencing protocol includes genomic DNA fragmentation, library

construction, cluster generation, and sequencing. The initial preparatory steps are the same for genome and exome libraries, wherein the fragmented genomic DNA is converted into an oligonucleotide adapter-tagged library. Multiple methods of genomic DNA fragmentation such as nebulization, sonication, restriction enzyme digestion, chemical methods, or sonication by adaptive focused acoustics result in DNA fragments about 200–600 base pair size range. All DNA fragments are then end repaired and ligated to platform-specific adapter oligonucleotides, which is capable of hybridizing to the oligonucleotides on the sequencer's flow cell surface, a thick glass fluidic device (Illumina Inc., San Diego, California). Before hybridizing, in the case of exome library enrichment, the libraries are hybridized with exome/target-specific capture probes. The captured libraries are further purified and enriched with exome kits and, in some protocols, amplified by limited cycles of polymerase chain reaction. Several exome capture/enrichment kits from different vendors are available. Though they differ in the targeted capture regions, capture probe length, probe composition, and performance characteristics, the most commonly used capture approach involves hybridization with Agilent SureSelect probe technology (Agilent Technologies, Santa Clara, California), followed by Roche Nimblegen Inc. (Madison, Wisconsin) and Illumina Inc. (San Diego, California).

The enriched libraries hybridized on the flow cell are then bridge amplified to generate clusters of DNA clones. Sequencing of clonal clusters proceeds in a cyclic manner with reversible dye-terminator chemistry, allowing only the first nucleotide base to be incorporated, that is, fluorescently labeled and reversibly terminated nucleotides. The high-sensitivity imaging optics are used to capture the fluorescent output of clusters on the flow cell after each base post incorporation. This process is repeated to yield a strand (read) whose length is dependent on the number of sequencing cycles. In paired-end sequencing, the same cluster is also sequenced from the opposite end, generating two reads per library. The sequencing of many samples can be run simultaneously by multiplexing. The libraries with adapters containing distinct index sequences, are used to identify the samples, can be pooled together in each run. Resulting reads made available in FASTQ format files are aligned to the human reference genome that are further run through computational pipelines for variant calling and subsequent downstream analysis.

Approximately 623 genes are known to be associated with eye disease (https://omim.org/) out of approximately 22,000 genes in the human genome. For WES, an enrichment approach needs to be strategically designed to provide complete coverage of known disease-associated genes as technically feasible for the coding regions and flanking intron-exon boundaries (e.g., 10–50 base pairs). Additionally, by careful characterization of capture efficiency and coverage using bioinformatic algorithms, specific deep intronic and untranslated regions associated with diseases can be included in the capture enrichment. Subsequently, the Sanger sequencing can be used to cover the low-coverage regions likely to be associated with the patient's clinical phenotype. In contrast to WES, without having capture probe enrichment, WGS results in less-biased sequence coverage that allows inspection of deeper intronic and regulatory regions. Further, the all structural variations are detected with specific bioinformatic algorithms. However, WGS still holds increase in sequencing cost and computational resources.

33.3 Bioinformatic Analysis for WES and WGS

Several open-source bioinformatic algorithms and commercial software exist to analyze WES and WGS large data files and process into variant call files (VCF) with high-quality variants, which require substantial computational resource and bioinformatic expertise. Each lab has unique pipeline made up of open-source, in-house developed, and/or commercial software; the WES pipeline we use is shown in Fig. 33.1. The bioinformatic analysis, although there are variations in the protocol, is a common multistep process for

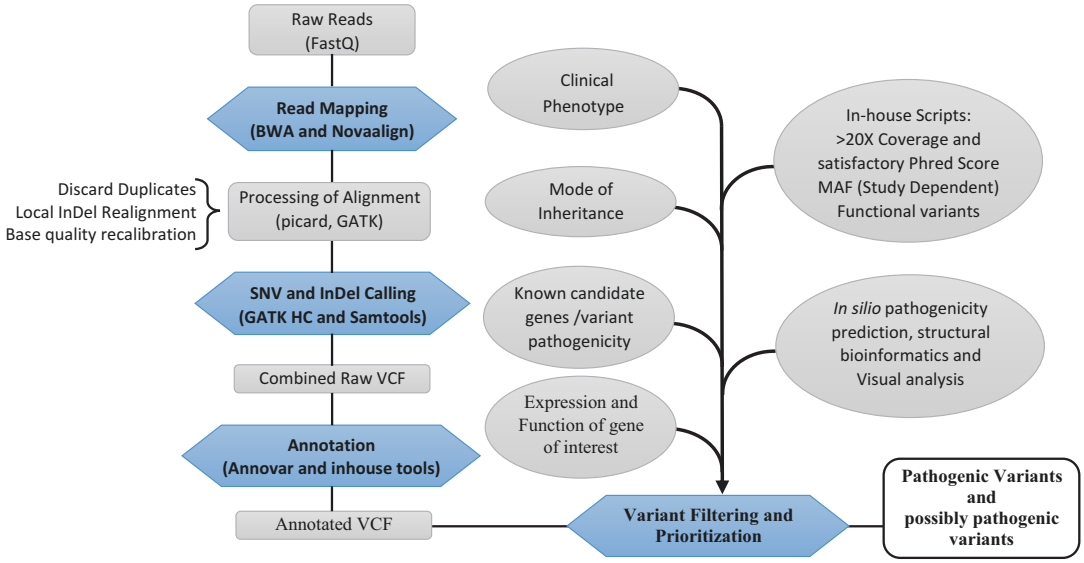

Fig. 33.1 A clinical exome sequencing analysis workflow for variant identification

WES and WGS. The primary analysis starts with the processes of initial alignment and mapping of sequence reads to the human genome reference in order to generate a binary alignment and mapping file after the quality control analysis of NGS data (Fig. 33.1). The secondary analysis is the variant preparation, involving variant calling and annotations, for the downstream clinical interpretation (tertiary analysis). The tertiary analysis includes variant incidence, prioritization, classification, and integration with the clinical features. Several reviews are referred here [9–11] for the primary and secondary analyses of WES and WGS data and have emphasized the sensitivity and accuracy of different algorithms in identification of variants. The tertiary analysis, a complex process, requires customized data-mining process for the identification of a disease-causing or disease-associated variant, which is depending on the phenotype.

33.3.1 Variant Filtering in Mendelian Diseases

WES/WGS have dramatically altered the traditional linkage-analysis landscape of finding rare variant in Mendelian disease, and we can now identify them in only the patient or patient with biological parents (trio) if they are available. The trio sequencing has been demonstrated as a preferred diagnostic approach, which allows for a more detailed analysis of variant inheritance patterns. A common first step in trio studies is to apply filters based on suspected disease inheritance patterns and disease frequency. In a presumed rare inherited disorder or general exome analysis workflow (Fig. 33.1), a common starting point is to filter common variants that are in the population assuming that rare variants are causative. This filtering step may differ based on the individual patients and laboratories. In common, variants with high minor allele frequency (MAF) equal to or greater than 1% in public databases will be removed. Several public databases include the 1000 Genomes Project (http://www.1000genomes.org/), ESP6500 (http://evs.gs.washington.edu/EVS/), dbSNP (http://www.ncbi.nlm.nih.gov/SNP/), ExAC (http://exac.broadinstitute.org/), and GenomeAD (http://gnomad.broadinstitute.org), which now serve as a valuable resource for MAF estimations. MAF cutoff around 1% and 0.5% or less may be used for rare recessive disorders and a dominant or X-linked disorder, respectively. Next, the remaining variants are filtered using the functional

impact of the variant. The truncating variants (stop gain/loss, start loss, or frameshift), missense variants, and canonical splice-site variants are considered first as protein function-altering variants, followed by silent and in-frame indels affecting protein-coding regions. Missense variants are further prioritized by their functional impact on the protein using in silico tools such as SIFT (http://sift.jcvi.org/) and Polyphen2 (http://genetics.bwh.harvard.edu/pph2/) and others [12]. This prioritized set is further stratified whether those are present in the same gene, those are predicted to be the most deleterious, and those are residing in the genes that are implicated in the patient's phenotype. Subsequent variant analysis is differed in trio sequencing compared to patient sequencing alone, wherein mode of inheritance is applied. For example, homozygous or compound heterozygous variants will be retained for recessive mode of inheritance whereas heterozygous variants for dominant mode of inheritance. For de novo variants, trio sequencing is highly recommended. On an average one to two de novo variants appear in a typical exome, which tend to be more deleterious than inherited variants, and are difficult to deduce from trio sequencing [13].

The detection of structural variations (SVs) including copy number variations (CNVs) may be treated separately, since the process of their detection differs substantially from the pipelines used to identify and small InDels and single nucleotide variants (SNVs). Detecting SVs has several challenges, and a number of approaches and commonly used methods include read depth, allele frequency, paired-end mapping, split-read mapping, and de novo assembly and have been reviewed elsewhere [14–16]. SVs, like other variants, can be common (polymorphisms with no clinical significance) or rare and potentially damaging, which often causes several clinical phenotypes. Using mode of inheritance, total rare SV burden, size, and number of dosage-sensitive genes in the SV region, causative SVs can be prioritized. In trio sequencing, SVs may present in patient alone compared to unaffected individuals, which helps to identify causative SVs. In addition, a number of public databases are available to annotate the potential clinical SVs. However, it is still challenging to determine the causative SVs. For example, causative CNVs may not be identical [17], and different SVs may be obtained with different technologies and bioinformatic approaches for a given sample. Regardless of these challenges, detection of SVs is important as their involvement in human eye diseases is increasingly being recognized.

Finally, a careful examination is required for the filtered variants prior to confirm their significance in the clinical practice, since more number of variants could be prioritized as potential causative variants through the above-described filtering methods. For example, the genome of healthy individual carries large number (at least 100 per genome) of loss-of-function variants, and yet they include rare disease-causing (causal) variants [18]. To distinguishing causal variants from the many potentially rare and functional variants that are commonly present, substantial time and effort must be taken to suggest causality. In presumed monogenic-disease cases, first one must evaluate genes and variant previously implicated in disease phenotypes before exploring novel. Second, the new gene can be implicated only when variants in the same gene and similar clinical features have been implicated in multiple individuals, along with unaffected controls [19], which requires considerable expertise and collaborative input from physician and genetician. Further genetic and functional studies are important to validate experimentally the predicted causal variants. Despite the current limitations of variant interpretation capabilities, the rapid pace of discovery in variant calling methods and new diseases-associated genes may help WES/WGS methods to achieve its clinical translation.

33.3.2 Variant Filtering in Complex Diseases

Prioritizing variants of WGS/WES data in a complex disease is very difficult and requires a different approach. GWAS has been used mostly for investigating the genetic architecture of complex diseases by following the common variant [MAF > 0.05]/common disease hypothesis [20].

In GWAS, several millions of single nucleotide polymorphisms (SNPs) are assayed in thousands of individuals with a phenotype and unrelated controls. However, challenges are still existing that most of the variants identified so far confer a small increase in risk and explain only a small proportion of familial clustering [20]. This missing heritability can be explained by the alternative hypothesis that rare [MAF < 0.01] and low-frequency [MAF 0.01–0.05] variants are likely to be strong effect and heritable [21]. WGS/WES are mostly employed to deduce rare and low-frequency variants that are likely to be causal in complex phenotypes. Thus far, the interpretations of causal variants can be challenging that the allelic architecture of complex diseases can be underpinned by a combination of spectrum of frequency and rare variants. For example, both common and rare variants have been identified in AMD patients using GWAS and NGS approaches [22, 23]. Currently, WGS is the preferred approach to exhaustively study the full allelic spectrum of variations in complex traits. The possible explanation is that rare variants in or near gene targets display larger average effects on phenotype, and both regulatory variants of comparable allele frequencies and common genetic variants are indeed causal [21, 24]. Thus, one must focus on the functional genomic approaches that include transcriptome and epigenome and along with association signal from GWAS that would help to understand their involvement in the complex phenotype. Even rigorous evaluation of each variant with functional consequences is required because the single variant displays small effect in complex disease. Several statistical methods have been developed to evaluate the effect of multiple variants that have functional consequences, most of them using variant aggregation approaches to address this issue. These methods can be classified into two main types as burden and variance component tests or a mixture of both [25]. Burden tests collapse the number of variants in a certain region or gene between cases and controls, while variance component tests distinguish variants with effect compared to variant with no effect in a single gene [25]. The overview and performance of currently available algorithms are reviewed elsewhere [26, 27].

33.4 Whole–Exome Sequencing in Eye Diseases

WES, primarily very successful in Mendelian disorders, has also offers in identifying causal variants in patients and families with complex diseases. In case of Mendelian disorders, screening the long list of known genes using targeted NGS does not reveal causal variants; for example, in most of the RD cases as described below, WES is proved to be efficient to discover novel causal variants and genes. In case of complex eye diseases such as cataract, glaucoma, diabetic retinopathy, age-related macular degeneration, and other degenerative eye disorders, it is difficult to identify causative variants due to the fact that it may be caused by combination of many genetic factors, and the involvement of environmental factors may affect the identification. Despite the fact, WES is still the preferred choice of method to identify causative variants. The major exome sequencing studies presented here were in the context of Asia-Pacific and the data is illustrated in Fig. 33.2.

33.4.1 Retinal Dystrophies

Retinal dystrophies (RD) contribute about 40% of all inherited eye diseases, and there are about 30 genes earlier reported to be associated with RD. Exome studies had been found to be powerful in identifying the molecular cause where the known genes did not show any mutation. In an Indian Muslim family, three out of eight members were affected with autosomal recessive cone dystrophy. WES showed a homozygous frameshift mutation leading to truncation in the affected members, and the same is found to be heterozygous in the unaffected family members in the CNGB3 gene [28]. In a consanguineous Israeli family with early-onset cone-rod dystrophy (CRD) and muscular dystrophy, exome sequencing and homozygosity mapping identified variants

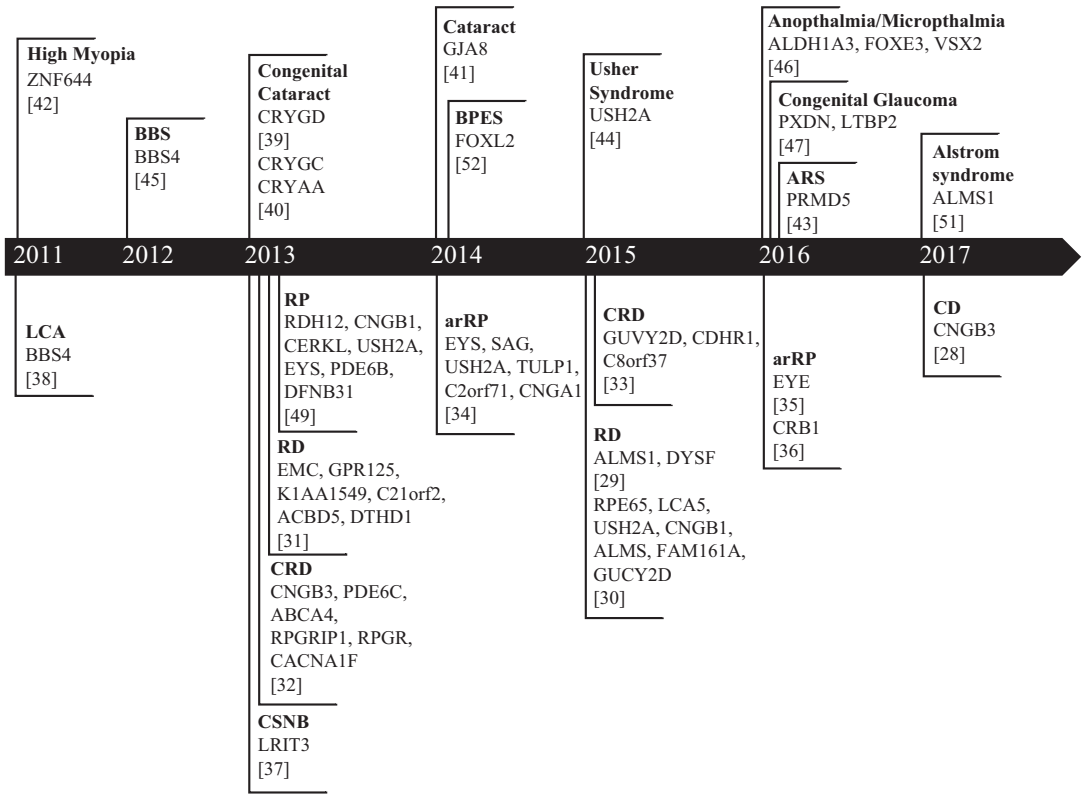

Fig. 33.2 Timeline of WES and WGS-based studies of eye diseases in Asian populations

in ALMS1 and DYSF that are linked genetically and physically on chromosome 2. Although both the genes were reported in Alstrom syndrome, the affected individuals did not have any symptoms other than mild muscular dystrophy [29].

In a study of 26 Pakistani families with inherited retinal degenerations, 9 causal mutations along with 6 novel variants in RPE65, LCA5, USH2A, CNGB1, FAM161A, CERKL, and GUCY2D were identified in 13 families. In addition to the causal variants, a total of 200 variants were also identified that are unique to Pakistani population as they were not reported in any of the genomic databases [30]. Genomic approaches including autozygome-guided mutation analysis and exome sequencing were performed in a large cohort of 150 families with retinal dystrophies. Six novel candidate disease genes (C21orf2, EMC1, KIAA1549, GPR125, ACBD5, and DTHD1) were identified in addition to causative genetic lesions in the known genes. Of the novel candidates, ACBD5 and DTHD1 were observed for the first time in the syndromic RD cases [31].

The power of exome sequencing was best witnessed in a study of the 47 Chinese families through the identification of potential pathogenic mutations in 10 families (21.28%) in the list of 25 known genes of retinal dystrophies. The earlier study from the same group had identified mutations in only 7 out of 130 families (5.38%) using the Sanger sequencing of complete exons in 5 genes and reported mutations in 17 genes [32]. In another study with cone-dominated retinopathy, WES followed by segregation analysis in six Israeli families revealed mutations in known retinopathy genes: GUCY2D gene in three families and one each in CDHR1 and C8orf37. Further targeted screening of additional cone-dominated families led to identification of GUCY2D mutations in four other families [33].

Retinitis pigmentosa (RP) is a heterogeneous group of inherited disorders characterized by

progressive degeneration of the retinal photoreceptor cells. In a large cohort study of Japanese RP patients, exome sequencing of 30 RP patients identified disease-causing gene mutations of CNGA1, EYS, and SAG along with potential disease-causing gene variants of USH2A, EYS, TULP1, and C2orf71. Further screening of most frequent CNGA1 gene mutation in 69 patients revealed 1 patient with a homozygous mutation [34]. Similarly, in a large cohort of 14 Indian autosomal recessive retinitis pigmentosa (arRP) families and 100 sporadic RP cases, a spectrum of novel EYS mutations including a frameshift mutation, 2 were stop-gain mutations, 1 was a splicing mutation, and the others were missense mutations that were identified in 2 families and 8 sporadic patients [35]. In another study of 2 Chinese families with 1 family having 3 affected members and 100 Indian sporadic families, exome sequencing revealed 4 novel mutations and 1 reported mutation in CRB1 gene, which has been known to cause severe retinal dystrophies [36].

Congenital stationary night blindness (CSNB) is a genetically heterogeneous retinal disorder inherited as either X-linked or autosomal recessive. Whole-exome sequencing in one simplex complete CSNB case which was negative for the known genes NYX, GRM6, TRPM1, and GPR179 led to the identification of a missense and a nonsense mutation in LRIT3 gene. Subsequent Sanger sequencing of 89 individuals showed a nonsense and a frameshift mutation in the same gene in another individual with complete CSNB [37]. WES was also employed in a consanguineous Saudi Arabian family with Leber congenital amaurosis (LCA) and identified a novel missense mutation in BBS4, which segregated with the disease. Further analysis in zebrafish also indicated that the mutation is pathogenic [38].

33.4.2 Cataract and Refractive Errors

Congenital cataract causing blindness in children is primarily inherited in an autosomal dominant manner. There are about 30 candidate genes suggested for this condition, and no mutations were identified by screening these known genes. Hence whole-exome sequencing with linkage analysis was used to identify the causative mutation in a Chinese family. Through linkage, 11 candidate mutations were selected, and finally CRYGD was found to be as the gene responsible for the cataract phenotype [39]. In another study of two Korean families with autosomal dominant congenital cataract (ADCC), a recurrent CRYAA missense mutation in family A and a novel CRYGC frameshift mutation in family B were identified [40]. Another exome study of proband with ADCC has found a recurrent missense mutation in GJA8 gene. This mutation was confirmed by Sanger sequencing and found to be co-segregating with the affected family members but was not detected in unrelated unaffected controls. This study demonstrates the utility of the exome sequencing in a clinical setup for identification of known and unknown disease-causing variants [41]. Exome analysis was also carried out in two affected individuals from a Han Chinese family with high myopia and identified a mutation in ZNF644 that was related to the phenotype. Further Sanger sequencing analysis of ZNF644 gene in 300 sporadic cases of high myopia detected 5 additional mutations in 11 different patients that were absent in 600 normal controls [42].

33.4.3 Ocular Syndromes

Axenfeld–Rieger syndrome (ARS; OMIM 180500) is a rare developmental disorder inherited in an autosomal dominant manner affecting the cornea, iris, lens, and angle, which is primarily caused by mutations in CYP1b1, PITX2, and FOXC1. A novel heterozygous missense variant in PRDM5 gene was identified in a proband with ARS, which co-segregates with the disease and found to be absent in matched controls and genomic databases [43]. In another study WES was performed in patients with Usher syndrome (USH), which is a multisensory degenerative disorder with deafness and blindness and genetically heterogeneous. A novel and two known

mutations in USH2A were detected in two affected patients that were absent in an unaffected relative and further confirmed by direct sequencing and co-segregation analysis [44]. WES also revealed a novel homozygous splice BBS1 mutation in four patients from two consanguineous Pakistani families with Bardet-Biedl syndrome, which is genetically heterogeneous disorder characterized by rod-cone dystrophy with other non-ocular features [45].

33.4.4 Other Eye Diseases

Anophthalmia and microphthalmia (A/M) are genetically heterogeneous disease caused by mutations in at least 20 genes that show different modes of inheritance. Exome sequencing of eight samples with anophthalmia and microphthalmia from Pakistani and Indian families identified three novel mutations including two mutations in ALDH1A3 gene and a missense mutation in FOXE3 gene. Additionally, two previously reported mutations were identified in FOXE3 and VSX2 [46]. In another study involving patients with primary congenital glaucoma (PCG), WES was performed in four individuals belonging to three different CYP1B1-negative Pakistani families and identified two mutations in the LTBP2 gene and one in the PXDN gene [47].

33.5 Whole-Genome Sequencing in Eye Diseases

The limitations of WES, include only 2% coverage of the human genome, missing pathogenic intronic variants that may affect transcripts, about 3 to 5% of missed exome targets, may not completely characterize copy number variations and breakpoints for other structural variants [25, 48], leads to unsolved cases. For example, approximately 20% of unsolved RP cases were detected as described previously [31]. In contrast, WGS shows evident in avoiding these limitations and offers complete exomic coverage and identification of noncoding pathogenic variations [48]. However, the data analysis of WGS is still very difficult. Thus far, only very few studies have been published to identify candidate variants in eye diseases. In the literature, we found only three studies of ocular diseases using WGS in Asian ethnic population. The first study published by Nishiguchi and colleagues performed WGS on 16 unrelated patients from North America or Japan with autosomal recessive retinitis pigmentosa (arRP) in search of pathogenic variants [49]. Homozygous or compound heterozygous mutations in seven genes (EYS, PDE6B, USH2A, CNGB1, CERKL, RDH12, and DFNB31) were detected. Of these, there was a 2.3 kb deletion in USH2A and an inverted duplication of ~446 kb in EYS, which would not be detected through WES. Moreover, a homozygous frameshift variant (p.L206 fs) in the ciliary gene NEK2 was identified as a new arRP gene [49]. In another study, whole-genome low-coverage sequencing (WGLCS) was used to characterize the breakpoints of blepharophimosis–ptosis–epicanthus inversus syndrome (BPES, OMIM 110100) and affects eyelid formation and ovarian function, in Han Chinese families. Four breakpoints were identified in the FOXL2 locus and show that disruption of FOXL2 gene can be accurately and rapidly detected using WGLCS [50]. Recently, WGS was performed on a Chinese quartet family with two siblings predominantly affected by cone-rod dystrophy and short stature to identify the full spectrum of the two siblings' genetic variations. Two compound heterozygous mutations (p.S1301X; p.R2146X) shared between two siblings in ALMS1 gene were identified [51]. Taken together, WGS substantially improves the detection of full spectrum of pathogenic variants in the eye diseases. With the costs of whole-genome sequencing continuously decreasing, WGS is now likely the preferred choice in the clinical settings.

33.6 Use of WES/WGS in the Clinical Settings

Compared to whole-genome sequencing, exome sequencing has become a widely used method for identifying the molecular basis of genetic eye

disorders, specifically to detect rare causal variants for Mendelian disorders. In recent years, WES has been successfully employed in the clinical settings, with the diagnostic rate of ~25% [52]. To test diagnostic rate in retinal dystrophies, WES and autozygosity-guided mutation analysis were performed in a large set of simplex and multiplex families with different RD phenotypes. The study showed WES is superior and also found six novel candidate genes. Molecular diagnostic rate of WES was 80% and 74% in simplex and multiplex cases, respectively, compared to 42% and 52% in simplex and multiplex cases by autozygome-guided sequencing [31]. However, recommendations of WES use in the clinical laboratories are still evolving with many challenges that delay the common use of WES in the molecular diagnosis. Mainly, the incomplete coverage of exonic regions due to sequence architecture (e.g., high G + C content) and evolving various variant detection and interpretation methods (e.g., copy number variants detection) limit the diagnostic rate. The evolution of genome information (e.g., http://gnomad.broadinstitute.org) may change the understanding of variant interpretation. Moreover, additional information from family studies and input from clinicians about patients may help to increase the diagnostic rate.

More recently, the use of WGS in clinical settings has been enabled. Although WGS is becoming more and more affordable and providing comprehensive view of genetic alterations, the bigger challenge of computational analysis of high volume of data exists. More importantly, individuals with high bioinformatic skills are needed for the analysis of data set since the constant change of bioinformatic analysis and algorithms along with the modifications of sequencing chemistries. Regardless, the use of NGS in the clinical settings provides a specific treatment or medical management that can have an impact on the clinical outcome. This poses unique challenges that include informed consent process, possibility of proband versus trio sequencing, cost and turnaround time, and how to interpret and communicate the results, including incidental findings and data sharing, to the patient and family and need for genetic counseling. The American College of Medical Genetics and Genomics (ACMG) has proposed recommendations on informed consent process, reporting of incidental findings and clinical laboratory standards for next-generation sequencing [53, 54]. However, individual laboratories may have their own policies for incidental findings, including which type of variant to be disclosed (e.g., pathogenic and possibly pathogenic). But then, their assay design should follow an adequate read depth and coverage, and accuracy in identifying variants includes confirmation of other orthogonal methods for CNVs and other structural variants.

33.7 Conclusions and Future Prospects

The presented literature here reveals that NGS-based genomic approaches to most of the eye diseases have so far been widely used and showed its utility in the clinical settings. However, WES-based methods showed its maximum utility in identifying novel causal variants and genes in eye diseases, especially in retinal degenerations. Regardless of its success, evolution of variant interpretation knowledge is required, and translating the findings into the clinical settings is a challenge in itself. Though the large amounts of data from both clinic and sequencing research projects are available, creation of dedicated loci-specific databases for eye diseases is on major need to enhance the process of variant interpretation. Indeed, new analysis methods to deal with various rare variants, discovered in millions of variants through WGS and WES approaches, into particular causal variants can have important implications in the disease management. In the near future, collaborative effort, combination of WGS and transcriptomic approaches, and the large cohort study may be of high priority to prioritize candidate variants for follow-up.

Conflict of Interest The authors declare no conflict of interest.

Compliance with Ethical Requirements No animal or human studies were carried out by the authors for this article.

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Myopia Genes in Asians

34

Shumin Tang, Yu Meng Wang, Aziz K. W. Kam, Tommy C. Y. Chan, Calvin C. P. Pang, Jason C. S. Yam, and Guy L. J. Chen

Abstract

Myopia is the most common ocular disorder causing visual impairment worldwide. It is a public health issue in many parts of the world. Compared with Caucasian or other ethnicities, its prevalence in Asians, especially Japanese, Koreans, and Chinese, is much higher. Environmental and genetic factors play important roles in myopia development. Myopia is a multifactorial disease. Time spent outdoors, amount of near work, and educational level influence myopia onset and progression. Recent advances in modern technology and molecular biology including linkage analyses, candidate gene analysis, genome-wide association studies (GWAS), whole-exome sequencing (WES), and next-generation sequencing (NGS) have led to mapping and identifying many myopia-associated gene loci and variants. Understanding the genetic basis may help in myopia prediction and prevention. This review is to summarize recent major findings in myopia genetics with a focus in Asian populations.

S. Tang · Y. M. Wang · T. C. Y. Chan · C. C. P. Pang · J. C. S. Yam · G. L. J. Chen (✉)
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, China
e-mail: lijia_chen@cuhk.edu.hk

A. K. W. Kam
Department of Ophthalmology and Visual Sciences, Prince of Wales Hospital, Hong Kong, China

Keywords

Myopia · Refractive errors · Genetics · Asians

34.1 Introduction

Myopia is the most common ophthalmic disorder posing risk to visual impairment worldwide [1, 2]. Its occurrence and severity are much more serious in East Asians than other ethnic populations, affecting as many as 90% of high school students in East Asia [1]. It is a public health problem. The longer axial length of myopic eyes is mismatched with the optical power of cornea and lens, focusing images in front of the retina, leading to blurred distant vision [3]. Although myopic refraction can be corrected by spectacles, contact lens, and/or refractive surgery, highly myopic eyes with elongated axial length and thinned sclera are at increased risk for vision-threatening complications, such as glaucoma, retinal detachment, choroidal neovascularization, macular hole, and myopic foveoschisis [4–7]. Therefore, the rapidly rising prevalence of myopia poses socioeconomic burden to individual and the society.

Both environmental and genetic factors play important roles in myopia development. Increased time spent outdoor reduces the incidence and progression of myopia in school children [8, 9]. Other factors include near work and

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education. In addition, more than 20 chromosomal loci and 100 genes have been identified to be associated with myopia or refractive errors via linkage analyses, candidate gene, genome-wide association studies (GWAS), next-generation sequencing, and whole-exome sequencing (WES) [10–23]. However, some of the gene associations are inconsistent among reported studies. So far no individual gene has been proven to solely account for the etiology of myopia. The mechanism of myopia development is complex and not fully explained. This review is to summarize the major findings in myopia genetics with a focus in Asian populations.

34.2 Ethnic Differences in Myopia Prevalence

Prevalence of myopia varies among different ethnicities with predominance in Asia. The Refractive Error Study in Children (RESC), employing standardized sampling strategies and measurement methods, determined the prevalence of refractive error in children across different ethnicities and cultures worldwide. Among children aged 5–15 years, the myopia prevalence was 21.6% in northern China [24] and higher at 38.1% in southern China [25]. In contrast, the prevalence was much lower at 7% in Chile and 4% in South Africa [26, 27]. In Australia, the Sydney Myopia Study (SMS) investigated children from grade 1 (6-year-old) to grade 7 (12-year-old), and the prevalence were 1.43% and 11.9%, respectively [28, 29]. Across the same age and using the same protocol as SMS in the Anyang County in northern China, the prevalence was 3.9% and 67.3%, respectively [30]. These population-based studies consistently suggested a higher prevalence of myopia in Asians.

34.3 Inheritability of Myopia

Family aggregation studies and twin studies indicated inheritability in myopia and supported the role of genetics in myopia development. Myopia heritability was reported up to 90% from twin studies [31, 32]. In addition, children with myopic parents are more likely to be myopic [33–35]. In 1994, Zadnik et al. revealed a longer eyeball length in those pre-myopic children with family history of myopia, when comparing to those without family history [36]. In 2008, our group further demonstrated that parental history of myopia would increase the growth rate of eyeball [37]. Children with two myopic parents had the greatest annual axial length (AL) growth of 0.37 mm, followed by children with one myopic parent (AL growth = 0.26 mm), and children with no myopic parents (AL growth = 0.20 mm) [37]. Both myopia onset and progression are affected by genetic factors.

34.4 The Myopia Loci

Many myopia loci have been discovered by linkage analysis. Linkage region of the entire genome was first identified, and that region was further analyzed by allelic association analysis. Till now, 25 myopia loci have been reported in the Online Mendelian Inheritance in Man (OMIM). Among them, 23 were located at the autosomal chromosome, and the other two were on the X-linked chromosome (Table 34.1).

34.4.1 *MYP1*

The first locus identified for high myopia, *MYP1*, was reported in 1990 and located at Xq28, inherited in X-linked recessive manner in a syndromic family with X-linked myopia, astigmatism, impaired vision, and optic nerve head hypoplasia [38]. In 2010, the X-linked recessive inheritance of *MYP1* locus was confirmed in a large non-syndromic family with high myopia [39]. However, causative mutation was not identified in the coding and adjacent intronic regions of *GPR50*, *PRRG3*, *CNGA2*, and *BGN*. In 2011, Ratnamala et al. refined the *MYP1* locus on Xq28 in the non-syndromic high myopia family and excluded 13 positional candidate genes as the high myopia causative genes in this region [40].

Table 34.1 Summary of reported myopia loci

Myopia loci	Related gene	Location	Phenotype	Ethnicity	Participants	MIM #	References
MYP1		Xq28	X-linked	Caucasian	1 family	310,460	[38]
MYP2		18p11.31	Autosomal dominant	Northern European and Chinese	8 families	160,700	[41, 42]
MYP3	*LUM, TGF1*	12q21-q23	Autosomal dominant	Italian/German	1 family	603,221	[45]
MYP5	*COL1A1*, *CHAD*	17q21-q22	Autosomal dominant	English/Canadian	1 family	608,474	[48]
MYP6	*SCO2*	22q12	Autosomal dominant	Jewish American	44 families	608,908	[49, 50]
MYP7	*PAX6*	11p13	Multifactorial	Caucasian	221 pairs of dizygotic twins	609,256	[51]
MYP8		3q26	Multifactorial			609,257	
MYP9		4q12	Multifactorial			609,258	
MYP10		8p23	Multifactorial			609,259	
MYP11	*RRH*	4q22-q27	Autosomal dominant	Chinese	1 family	609,994	[52]
MYP12	*SAG*, *DGKD*	2q37.1	Autosomal dominant	Northern European	1 family	609,995	[53]
MYP13		Xq23-q27.2	X-linked	Chinese		300,613	[54]
MYP14		1p36	NA	Ashkenazi Jewish	49 families	610,320	[55]
MYP15	*PCDH15*, *ZWINT*	10q21.1	Autosomal dominant	Caucasian	1 family	612,717	[56]
MYP16		5p15.33-p15.2	Autosomal dominant	Chinese	3 families	612,554	[57]
MYP17, MYP4		7p36; 7p15	Autosomal dominant	French and Algerian; African-American	23 families; 96 families	608,367	[58–60]
MYP18		14q22.1-q24.2	Autosomal recessive	Chinese	1 family	255,500	[61]
MYP19		5p15.1-p13.3	Autosomal dominant	Chinese	1 family	613,969	[62]
MYP20		13q12.12	Autosomal dominant	Chinese	419 high myopia and 669 controls	614,166	[20]
MYP21	*ZNF644*	1p22.2	Autosomal dominant	Chinese	1 family	614,159	[63]
MYP22	*CCDC111*	4q35.1	Autosomal dominant	Chinese	1 family	615,420	[64]
MYP23	*LRPAP1*	4p16.3	Autosomal recessive	Saudi Arabian	3 families	615,431	[65, 66]
MYP24	*SLC39A5*	12q13.3	Autosomal dominant	Chinese	1 family	615,946	[67]
MYP25	*P2HA2*	5q31.3	Autosomal dominant	Chinese	A three-generation family	617,238	[68]

34.4.2 *MYP2*

The *MYP2* locus was identified on 18p by Young et al. in 1998 through a genome-wide scan for myopia loci in eight multigenerational high myopia families with autosomal dominant pattern; one of the pedigrees was ethnic Chinese [41, 42]. In 2003, our group investigated the coding exons of the transforming growth factor-beta-induced factor (*TGIF*) on the *MYP2* locus by screening the DNA sequence among 71 high myopia subjects and 105 control subjects, all Hong Kong Chinese. Six single-nucleotide polymorphisms (SNPs) showed a significant difference between patients and controls, and four of them caused codon changes, suggesting *TGIF* as a susceptibility gene for high myopia [43]. In 2005, Scavello et al. studied nine candidate genes on the *MYP2* locus in a Caucasian cohort but did not identify any alteration on these genes in high myopia subjects [44].

34.4.3 *MYP3*

The *MYP3* locus at 12q21-q23 was identified in a large German/Italian family of high myopia with autosomal dominant inheritance [48]. *Decorin* on 12q23 and *lumican* (*LUM*) on 12q21.3-q22 were suggested as candidate genes, in view of their roles in coding the proteoglycan proteins, an important component of the extracellular matrix of sclera [45]. However, the *lumican* gene could not be identified on the *MYP3* locus in their subsequent study [46]. In 2010, Lin et al. detected the haplotype of four SNPs at the promoter region of *LUM* gene, which was significantly different between high myopia subjects and controls in a Taiwan Chinese population. A novel SNP of *LUM* (c.1567 C/T) associated with high myopia was identified [47].

34.4.4 Other Myopia Loci

The *MYP5* locus on 17q21-q22 was identified in a multigenerational English/Canadian family with autosomal dominant pattern of severe myopia. The extracellular matrix protein *COL1A1* and *chondroadherin* (*CHAD*) on 17q were proposed as potential candidate genes for high myopia [48].

The *MYP6* locus on 22q12 region was discovered among 44 Ashkenazi Jewish families with myopia less than −1.0D by performing the genome-wide linkage scan [49]. *SCO2* gene on 22q12, which codes for a copper homeostasis protein in the mitochondrial cytochrome *c* oxidase activity, has been identified for high myopia in an 11-member family with the autosomal dominant inheritance [50].

The *MYP7*, *MYP8*, *MYP9*, and *MYP10* loci have been identified by Hammond et al. who performed a genome-wide scan in 221 dizygotic twin pairs with different refractions. Totally four loci were observed at chromosomes 11p13 (*MYP7*), 3q26 (*MYP8*), 4q12 (*MYP9*), and 8p23 (*MYP10*). As the *PAX6* gene was located on the *MYP7* locus, the tagging SNPs showed strong evidence of linkage, suggesting a role of *PAX6* in myopia [51].

MYP11 on chromosome 4q22-q27 was identified in an autosomal dominant Chinese family of 12 members affected with high myopia. Retinal pigment epithelium-derived rhodopsin homolog gene (*RRH*), located on the *MYP*11 locus, was excluded as a causative gene, as subsequent sequence analysis did not identify any causative mutations [52].

MYP12 on chromosome 2q37.1 was identified in a large US family originated from Northern Europe with high myopia in an autosomal dominant pattern. However, sequencing analyses of the potential candidate genes in the region, S-antigen (*SAG*) and diacylglycerol kinase-delta (*DGKD*), did not find any causative mutations [53].

MYP13 was identified in a four-generation Chinese family of high myopia with X-linked inheritance, mapping to Xq23-q25. In addition to high myopia, the affected individuals had reduced permanent visual acuity and typical high myopic fundal changes [54].

MYP14 on chromosome 1p36 was detected from 49 multigenerational Ashkenazi Jewish families by conducting quantitative trait locus linkage analysis [55].

MYP15 was mapped to 10q21.1 among a large Hutterite family *from* South Dakota segregating high myopia [56]. Protocadherin 15 (*CDH15*) and ZWIO interactor (*ZWINT*), within the linkage region, were screened, but no causative mutation was found.

MYP16 was identified by our group when we completed one of the first genome-wide scans in Asians, on the chromosome 5p15.33-p15.2, in three Hong Kong Chinese pedigrees with autosomal dominant high myopia [57].

Naiglin et al. found *MYP4* locus on chromosome 7q36 in 21 French families and 2 Algerian families with autosomal dominant high myopia in 2002 [58]. Subsequently in 2008, Paget et al. studied 26 families and demonstrated significant linkage to chromosome 7p15 named *MYP17* in a nonparametric model [59]. Ciner et al. reported results of a QTL linkage analysis for ocular refraction in 96 African-American families with myopic probands to chromosome 7p15 [60].

MYP18 on chromosome 14q22.1-q24.2 was identified from a consanguineous Chinese family with autosomal recessive high myopia vis genome-wide linkage analysis [61].

MYP19 was identified on chromosome 5p15.1-p13.3 in a linkage analysis in a four-generation Chinese family segregating autosomal dominant high myopia [62].

MYP20, unlike the previously reported loci, was identified in a genome-wide association study of 493,947 SNPs in 419 Han Chinese individuals with high myopia and 669 unrelated controls. The SNP rs9318086 at 13q12.12 showed significant association with high myopia (P = 1.91E-16). The genes *MIPEP*, *C1QTNF9B-AS1*, and *C1QTNF9B* were at the locus, and two of them (*MIPEP* and *C1QTNF9B*) were expressed in the retina and retinal pigment epithelium [20].

MYP21 on chromosome 1p22.2 was identified by exome sequencing and segregation analysis in a five-generation Han Chinese family with autosomal dominant inheritance of high myopia, with a missense mutation in the *ZNF644* gene, which was expressed in human retinal and retinal pigment epithelium [63].

MYP22 was identified in a four-generation Chinese family with autosomal dominant high myopia. Exome sequencing identified a missense mutation in the *CCDC111* gene that was segregated with disease in the family while absent in 270 Chinese controls [64].

MYP23 was identified in three consanguineous Saudi Arabian families of non-syndromic extreme myopia by exome sequencing, which revealed two homozygous truncating mutations in the *LRPAP1* gene on chromosome 4p16.3. Linkage analysis showed one peak on chromosome 4 with a LOD score of 7 [65]. Subsequently, *LRPAP1* gene mutations were identified in 298 Chinese families with early-onset high myopia [66].

MYP24 was found in a three-generation Chinese family segregating autosomal dominant high myopia by whole-genome linkage analysis and exome sequencing, with *SLC39A5* mutations in high myopia individuals [67].

MYP25 was identified in a three-generation Chinese family segregating with autosomal dominant high myopia by whole-exome sequencing. A missense mutation in the *P4HA2* gene on chromosome 5 was found in 5 high myopic members of the family, but not in 626 controls [68].

It is notable that starting from MYP16 reported in 2008, eight myopia loci were identified in Chinese pedigrees.

34.5 Myopia Genes Identified from GenomeWide Association Studies (GWAS)

Since 2009, a total of 13 GWAS have been reported for myopia, refractive errors, and axial length (AL) (Table 34.2). Seven of them investigated genetic variants for high myopia or myopia, of which six were conducted in Asians and one in Caucasians. In 2009, Nakanishi et al. reported the first GWAS for myopia. SNP rs577948, within 200 kb DNA encompassed by *BLID* and *LOC399959*, was associated with high myopia in Japanese [18]. In 2011, rs10034228 in *MYP11* was associated with high myopia in a Chinese cohort [17]. Shi and our group reported

Table 34.2 Genes identified for myopia, refractive errors, and axial length from genome-wide association studies

Authors	Years	Sample size		Disease/phenotype	Ethnicity	SNP	Chromosome	Related gene	OR	P value (pooled)	References
		Case	Control								
Nakanishi et al.	2009	839	1914	High myopia	Japanese	rs577948	11	*BLID, LOC399959*	1.37(1.21–1.54)	2.22E–07	[18]
Li et al.	2011	2891	10,071	High myopia	Chinese	rs10034228	4	*MYP11*	0.81(0.76–0.86)	7.70E–13	[17]
Shi et al.	2011	3222	6311	High myopia	Chinese	rs9318086	13	*MIPEP*	1.64(1.46–1.85)	1.91E–16	[20]
Fan et al.	2012	4944		AL/high myopia	Chinese, Malay	rs4373767	1	*ZC3H11B*	0.75(0.68–0.84)	2.69E–10	[11]
Khor et al.	2013	1603	3427	High myopia	Singapore Chinese, Japanese	rs13382811	2	*ZFHX1B*	1.33	7.44E10–9	[13]
						rs6469937	8	*SNTB1*	0.75	6.08E10–8	
Shi et al.	2013	2758	4605	High myopia	Han Chinese	rs2730260	7	*VIPR2*	1.77(1.47–2.14)	8.95E–14	[19]
		2741	4599			rs4455882	8	*SNTB1*	0.59(0.47–0.73)	2.13E–11	
Kiefer et al.	2013	25,999	19,772	Myopia	European	rs12193446	6	*LAMA2*	0.79(0.76–0.81)	1.4E10–45	[14]
						rs1381566	11	*LRRC4C*	1.15(1.12–1.18)	3E10–26	
						rs17648524	16	*RBFOX1*	1.1(1.08–1.12)	1.3E10–22	
						rs7744813	6	*KCNQ5*	0.91(0.89–0.93)	6.6E10–22	
						rs3138142	12	*RDH5*	0.89(0.87–0.91)	1.8E10–20	
						chr8:60178580	8	*TOX/CA8*	0.91(0.90–0.93)	3.5E10–19	
						rs524952	15	*GOLGA8B/GJD2*	1.09(1.07–1.11)	5.6E10–19	
						rs2137277	8	*SFRP1*	0.9(0.88–0.92)	4.7E10–16	
						rs1550094	2	*PRSS56*	1.09(1.07–1.11)	1.3E10–15	
						rs2908972	17	*SHISA6*	1.07(1.06–1.09)	4.5E10–13	
						rs17412774	2	*PABPCP2*	0.93(0.92–0.95)	1.1E10–12	
						rs11145746	9	*TJP2*	1.09(1.06–1.11)	2.3E10–11	
						rs28412916	15	*RASGRF1*	1.07(1.05–1.09)	3.5E10–11	
						rs5022942	4	*BMP3*	1.08(1.05–1.10)	1.4E10–10	
						rs745480	10	*RGR*	1.06(1.04–1.08)	2.5E10–10	
						rs2155413	11	*DLG2*	1.06(1.04–1.08)	4.7E10–10	
						rs13091182	3	*ZBTB38*	0.94(0.92–0.96)	9E10–10	
						rs17400325	2	*PDE11A*	1.14(1.10–1.19)	1.9E10–9	
						rs17428076	2	*DLX1*	0.94(0.92–0.96)	2.8E10–9	
						rs6480859	10	*KCNMA1*	1.06(1.04–1.08)	1.2E10–8	
						chr14:54413001	14	*BMP4*	0.95(0.93–0.96)	1.7E10–8	

					rs4291789	13	*ZIC2*	1.07(1.05–1.09)	2.1E10–8	
								Beta (for quantitative trait)		
Cheng et al.	2013	20,747	AL	European, Asian	rs4074961	1	Intron 4 of *RSPO1*	0.07	4.00E–13	[10]
					rs994767	1	7 kb upstream of *ZC3H11B*	−0.07	9.60E–12	
					rs9811920	3	Intron 1 of *C3orf26*	0.08	4.90E–11	
					rs12193446	6	Intron 58 of *LAMA2*	0.12	1.20E–08	
					rs11073058	15	57 kb upstream of *GJD2*	0.07	4.30E–11	
					rs12321	22	*3′ UTR of ZNRF3*	−0.05	4.10E–08	
Hysi et al.	2010	17,684	Refractive errors	Caucasian	rs939658	15	*RASGRF1*	−0.15	1.85E–09	[12]
Solouki. et al.	2010	15,608	Refractive errors	Caucasian	rs634990	15	*GJD2 and ACTC1*	−0.23	2.21E–14	[21]
Verhoeven et al.	2013	45,758	Refractive errors	European, Asian	rs1652333	1	*CD55*	−0.112	3.05E10–12	[23]
					rs1656404	2	*PRSS56*	−0.153	7.86E10–11	
					rs1881492	2	*CHRNG*	−0.139	5.15E10–11	
					rs14165	3	*CACNA1D*	0.10	2.14E10–8	
					rs1960445	4	*BMP3*	−0.114	1.25E10–6	
					rs12205363	6	*LAMA2*	0.24	1.79E10–12	
					rs4237036	8	*CHD7*	0.09	1.82E10–8	
					rs7837791	8	*TOX*	0.11	3.99E10–12	
					rs7829127	8	*ZMAT4*	0.12	3.69E10–10	
					rs7042950	9	*RORB*	−0.096	4.15E10–8	
					rs10882165	10	*CYP26A1*	−0.107	1.03E10–11	
					rs7084402	10	*BICC1*	−0.108	2.06E10–13	
					rs11601239	11	*GRIA4*	−0.095	5.92E10–9	
					rs3138144	12	*RDH5*	0.12	4.44E10–12	
					rs2184971	13	*PCCA*	0.09	2.11E10–8	
					rs8000973	13	*ZIC2*	0.08	5.1E10–8	
					rs524952	15	*GJD2a*	−0.158	1.44E10–15	

(continued)

Table 34.2 (continued)

Authors	Years	Sample size		Disease/phenotype	Ethnicity	SNP	Chromosome	Related gene	OR	P value (pooled)	References
		Case	Control								
						rs4778879	15	*RASGRF1a*	−0.102	4.25E10–11	
						rs17183295	17	*MYO1D*	−0.131	9.66E10–11	
						rs4793501	17	*KCNJ2*	0.08	2.79E10–8	
						rs12971120	18	*CNDP2*	0.10	1.85E10–7	
Stambolian et al.	2013	26,953		Refractive errors	European ancestry	rs10500355	16	*RBFOX1*	−0.11	3.90E–09	[22]
Fan et al.	2016	50,356		Refractive errors	European, Asian	rs60843830	2	*FAM150B-ACP1*	−0.10	1.27E10–9	[69]
						rs10946507	6	*LINC00340*	−0.08	2.24E10–8	
						rs8023401	15	*FBN1*	−0.13	2.85E10–9	
						rs16949788	15	*DIS3L-MAP2K1*	−0.13	2.19E10–8	
						rs10880855	12	*ARID2-SNAT1*	−0.09	4.38E10–8	
						rs10853531	18	*SLC14A2*	−0.11	2.54E10–8	

a high myopia-associated SNP rs9318086 in a combined Chinese cohort. The locus contains three genes, *MIPEP*, *C1QTNF9B-AS1*, and *C1QTNF9B* [20]. A GWAS in Singapore including Chinese and Malays revealed the protective effect of the minor C allele of rs4373767 on *ZC3H11B* against high myopia and longer AL. The neighboring genes *SLC30A10* and *LYPLAL1* were expressed in the retinal pigment epithelium and sclera [11]. A genome-wide meta-analysis on high myopia and 286,031 SNPs in a combined Han Chinese cohort of 665 cases and 960 controls identified two myopia-associated genes, *VIPR2* and *SNTB1* [19]. Khor et al. found association of SNP rs13382811 in *ZFHX1B* with severe myopia in Chinese and Japanese [13]. In 20 genetic regions associated with the age of myopia onset, 16 of them were near to genes involved in eye development in Caucasian [14].

In addition to high myopia, the myopia-related quantitative traits including refractive errors and AL have been investigated in six GWAS or genome-wide meta-analyses. SNP rs8027411 on 15q25 was associated with refractive errors in Caucasians [12]. Another Caucasian study identified a myopia locus at chromosome 15q14, where the *GJD2* and *ACTC1* genes were found to express in the retina and sclera in animal models [21]. A genome-wide meta-analysis among multi-ancestry cohorts including 37,382 European and 8376 Asians reported 18 refractive error-associated SNPs [23]. Meta-analysis of the GWAS data of five European cohorts identified association of *RBFOX1* rs10500355 with refractive error [22]. *RBFOX1* was involved in neuronal development and maturation. In a study involving both European and Asian study subjects, eight loci were associated with axial length, and four of them also associated with refractive errors [22]. Recently, Fan et al. conducted a meta-analysis of gene-environment-wide association scans for refractive errors in European and Asian myopia subjects, including the SNP-education interaction. Six loci for refractive error have been identified in the European ancestry individuals and three in the Asians. Notably, the Asian loci showed significant interaction with education level, but this effect was less evident in Europeans [69].

34.6 Myopia Candidate Genes in Asians

A number of myopia candidate genes have been suggested from various association studies. We herein categorize these genes into sclera-related genes, growth factor-related genes, master control genes, muscarinic-related genes, and other genes (Table 34.3).

34.6.1 Sclera-Related Genes: Matrix Metallopeptidase Genes, Collagen-Related Genes, and Proteoglycan-Related Gene

In myopes, the axial length is elongated, and sclera is stretched resulting in its thinning and remodeling. Sclera is comprised of extracellular matrix, matrix-secreting fibroblasts, collagen, and proteoglycans. Scleral remodeling is believed to be a critical determinant in the development of myopia and is mediated by increasing collagen degradation and decreasing collagen and proteoglycans production to reduce extracellular matrix production [70]. The vitreal concentration of matrix metallopeptidase 2 (MMP2) was found to be higher in high myopic eyes than non-myopic eyes [71]. Animal studies showed that the scleral MMP expression was increased in form-deprivation myopic tree shrews and in guinea pigs [72–75]. *MMP1* and *MMP2* genes were found to be associated with refractive error in Amish families [76] but not in Asians. Scleral tissue contains about 90% of collagen by weight [77]. Collagen, type I, alpha 1 (*COL1A1*) gene was shown to be associated with high myopia in Chinese and Japanese populations [78, 79]. Proteoglycans are other major components of the scleral extracellular matrix to regulate collagen fibril assembly and interaction [80]. The *LUM* gene is a member of small leucine-rich proteoglycan (*SLRP*) gene family [80]. Proteoglycans are major components of the scleral extracellular matrix, which plays an important role in regulating collagen fibril assembly and interaction, and are intensely related to the structure and

Table 34.3 Candidate genes for myopia in Asians

Gene symbol	Gene name	Location	Ethnicity	Phenotype	References
Collagen-related genes					
COL1A1	*Collagen type I alpha 1*	17q21.33	Chinese, Japanese	High myopia (≤-6D)	[78, 79]
Proteoglycan-related genes					
LUM	*Lumican*	12q21.33	Chinese	High myopia (≤-10D)	[47, 81, 82]
Growth factor-related genes					
TGFB1	*Transforming growth factor-beta 1*	19q13.2	Chinese	High myopia (≤-6D)	[85, 86]
TGFB2	*Transforming growth factor-beta 2*	1q41	Chinese	High myopia (≤-6.5D)	[87]
TGIF	*Transforming growth factor-beta-induced factor*	18p11.31	Chinese	High myopia (≤-6D)	[43]
HGF	*Hepatocyte growth factor*	7q21.11	Chinese	High myopia (≤-10D)	[89]
MET	*Hepatocyte growth factor receptor*	7q31.2	Chinese	High myopia (≤-6D)	[90]
IGF1	*Insulin-like growth factor 1*	12q23.2	Chinese	High myopia (≤-9D)	[92]
Master control genes					
PAX6	*Paired box 6*	*11p13*	Chinese, Japanese	High myopia (≤-6D)	[100–104]
Muscarinic-related genes					
CHRM1	*Cholinergic receptor muscarinic 1*	*11q12.3*	Chinese	High myopia (≤-6.5D)	[107]
CHRM2	*Cholinergic receptor muscarinic 2*	*7q33*	Chinese	High myopia (≤-6D)	[108]
CHRM3	*Cholinergic receptor muscarinic 3*	*1q43*	Chinese		
CHRM4	*Cholinergic receptor muscarinic 4*	*11p11.2*	Chinese		
Others					
LAMA1	*Laminin, alpha 1*	*18p11.31-p11.23*	Chinese	High myopia (≤-6D)	[109]
UMODL1	*Uromodulin-like 1*	21q22.3	Japanese	High myopia (≤-9.25D)	[110]
CRYBA4	*Crystallin beta A4*	*22q12.1*	Chinese	High myopia (≤-8D)	[111]
BMP2K	*BMP2-inducible kinase*	*4q21.21*	Chinese	High myopia (≤-6D)	[112]
MYOC	Myocilin	1q24.3	Chinese	High myopia (≤-6D)	[113]

function of the sclera [80]. *LUM* polymorphisms were found to be associated with high myopia in Chinese populations [47, 81, 82].

34.6.2 Growth Factor-Related Genes

Ocular elongation involves active scleral growth [83]. Growth factors, such as transforming growth factor-beta 1 (TGFB1), are expressed in ocular tissues to regulate fibroblast proliferation and collagen production of sclera. Animal studies showed that isoform-specific changes in TGFB1 resulted in a decrease in collagen synthesis of sclera [84]. *TGFB1* has been reported as a susceptibility gene for high myopia in Chinese populations [85, 86]. Another scleral structure-related gene, *TGFB2* gene, was also associated with the development of high myopia in Chinese [87]. The aqueous concentration of TGFB2 was higher in Chinese subjects with longer axial length [88]. In addition, our group identified the transforming growth factor-beta-induced factor (*TGIF*) gene as a candidate gene for high myopia in Chinese [43].

Hepatocyte growth factor (HGF) is an important multifunctional cytokine for cellular

scattering and proliferation. Han et al. found the association of *HGF* gene with high myopia in a Han Chinese population [89]. Furthermore, the hepatocyte growth factor receptor (*MET*) gene was also associated with high myopia in Chinese [90]. Animal studies have demonstrated the role of insulin and insulin-like growth factor 1 (IGF1) in retinal development and ocular growth [91]. The *IGF1* gene was associated with high myopia in Chinese adults [92]. This association was replicated in the Egyptians [93] but not in other Asian populations [94–96].

34.6.3 *PAX6*

The paired box 6 (*PAX6)* gene is a member of the paired-domain Pax family. It regulates tissue-specific expression of diverse molecules, including transcription factors, cell adhesion molecules, hormones, and structural proteins [97]. The PAX6 protein is a transcriptional factor, regulating the eyeball development [98, 99]. In 2004, Hammond et al. first revealed *PAX6* as a susceptibility gene for myopia [51]. Later, two Chinese family studies showed the presence of *PAX6* variants in extreme refractive error [100]. Our group found that the AC and AG dinucleotide repeats in the *PAX6* P1 promoter affected its transcription activities and was associated with high myopia [101]. SNP rs662702 at the 3′ untranslated region (UTR) of *PAX6* was reported as a risk marker for extreme myopia in a Taiwan Chinese study [102]. The *PAX6* haplotypes GTAA, AGTG, and AGTA, defined by rs2071754, rs3026393, rs1506, and rs12421026, were associated with high myopia [103]. Meta-analysis of the published data confirmed association of *PAX6* rs644242 with extreme and high myopia [104].

34.6.4 Muscarinic Related and Other Genes

Animal and clinical studies have showed the effect of atropine, a muscarinic acetylcholine antagonist, in retarding axial elongation and myopia development [105, 106]. In Chinese, high myopia is associated with the muscarinic acetylcholine receptor genes *CHRM1*, *CHRM2*, *CHRM3*, and *CHRM4* [107, 108]. Other myopia-associated genes identified in Asian populations include laminin alpha 1 (*LAMA1*) [109], uromodulin-like 1 (*UMODL1*) [110], crystallin beta A4 (*CRYBA4*) [111], BMP2-inducible kinase (*BMP2K*) [112], and myocilin (*MYOC*) [113].

34.7 Rare Variants in Asians

The advent of advanced and high-throughput genomic technologies, specifically genome-wide association studies (GWAS), whole-exome sequencing (WES), and next-generation sequencing, provide important tools to discover gene variants for myopia or refractive errors. In 2011, Shi et al. first identified causative mutations in *ZNF644* among 11 Chinese high myopic patients by exome sequencing [63]. In 2013 a novel missense variant of the *CCDC111* gene was discovered for high myopia in Chinese patients [64]. *SLC39A5* mutations regulating the *BMP/TGF-β* pathway were identified in a Chinese high myopia family and a sporadic patient [67]. A mutation of the *LEPREL1* gene was identified in an autosomal-recessive high myopia family with co-existing early-onset cataract. *LEPREL1* is involved in the collagen modifications in the eye development [114]. Another causative gene for high myopia, *P4HA2*, was also identified by the same group [68]. Jiang et al. identified a homozygous frameshift mutation in *LRPAP1* gene in a Chinese consanguineous family with high myopia [66]. Frameshift mutations in *LOXL3* were identified in two of the 298 probands with early-onset high myopia [115]. In 2017, Jin et al. pioneered a whole-exome study for high myopia in 18 unrelated Chinese trios. All probands had early-onset high myopia before the age of 6 with both parents non-myopic. The results revealed de novo mutations in the *BSG* gene in early-onset high myopia [116].

34.8 Gene-Environment Interaction

Both genetic and environmental factors contribute to myopia development in all studied populations over the world. Independent effect of each factor is not possible to be separated from each other. Interactions are involved. For example, children with myopic parents are more likely to be raised in myopiogenic environment than those with non-myopic parents. Gene-environment interaction studies have been reported recently [69, 117]. Joint meta-analysis approach on SNP main effects and SNP-environment interactions was conducted [118]. Fan et al. investigated the interaction effect of education with those SNPs identified from previous GWAS on refractive error in Chinese, Malay, and Indian. Three genetic loci *SHISA6-DNAH9*, *GJD2*, and *ZMAT4-SFRP1* exhibited a strong association with myopic refractive error in individuals with higher secondary or university education. Low level of education may attenuate the effect of risk alleles on myopia [117]. The Consortium for Refractive Error and Myopia (CREAM) also performed a joint meta-analysis to test gene-education interaction effects on refractive error in both European and Asian groups. Six novel refractive error-associated loci (*FAM150B-ACP1*, *LINC00340*, *FBN1*, *DIS3L-MAP 2 K1*, *ARID2-SNAT1*, and *SLC14A2*) were identified in European group and three loci (*AREG*, *GABRR1*, and *PDE10A*) in Asian group. It was observed that gene-education interaction was significant in the Asian loci, but not in the European loci [69]. Gene-environment interaction may contribute to the heterogeneity of myopia.

34.9 Conclusive Remarks

Myopia is the commonest ocular disorders worldwide, especially in East Asia. Recent scientific and technological advancements including linkage analyses, candidate genes and genome-wide association studies (GWAS), and next-generation sequencing (NGS) have led to identification of a large number of myopia-associated gene loci and variants. However, currently none of the reported genes has been shown to account for even a modest fraction of risk of myopia. Furthermore, inconsistency of data exists among various studies. The intrinsic genetic constitutions and external environmental factors that lead to the strikingly high prevalence and severity of myopia in East Asians as compared with other ethnic populations have to be explored and understood through vigorous and large-scale research with multicenter efforts. Replication and aggregation cohort studies to confirm the true association are necessary. Functional studies on identified genes should be conducted to throw light on the mechanism of myopia. The role of environmental factors to genetic influences should be further explored to explain the heterogeneity of myopia development.

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Keratoconus Genes in Chinese

35

Yu Meng Wang, Ka Wai Kam, Tommy C. Y. Chan, Alvin L. Young, Vishal Jhanji, Guy L. J. Chen, and Calvin C. P. Pang

Abstract

Keratoconus is a corneal disease characterized by conical protrusion and progressive thinning of the cornea, resulting in various degrees of visual impairment. Keratoconus affects all ethnic groups, but the prevalence is higher among the Asian populations. The onset of keratoconus is insidious and often occurs during late childhood. Early diagnosis is difficult. If untreated, the disease often progresses irreversibly and can lead to blindness. Nowadays, corneal collagen cross-linking has shown some promising results in retarding or halting keratoconus progression, but currently there is no complete cure. Keratoconus is a multifactorial disease resulting from the interaction of environmental, behavioral, and genetic factors. Its progression has been associated with structural, biochemical, cellular, and molecular alternations in corneal collagen lamellae, higher systemic oxidative stress, and modifications in corneal proteins. However, the etiology of keratoconus remains unclear and the exact regulatory mechanism still elusive. There is evidence of familial aggregation, monozygotic twin concordance, association with other genetic diseases, and the ethnic difference in prevalence and incidences. This chapter attempts to summarize the current knowledge and research of keratoconus epidemiology, pathology, and genetics, with a particular focus for studies in the Chinese population.

Keywords

Keratoconus · Genetics · Epidemiology · Pathology · Chinese

Y. M. Wang · T. C. Y. Chan · C. C. P. Pang (✉)
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong Eye Hospital, Kowloon, Hong Kong
e-mail: cppang@cuhk.edu.hk

K. W. Kam · A. L. Young · G. L. J. Chen
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong Eye Hospital, Kowloon, Hong Kong

Department of Ophthalmology and Visual Sciences, Prince of Wales Hospital, Shatin, Hong Kong

V. Jhanji
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong Eye Hospital, Kowloon, Hong Kong

UPMC Eye Center, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA

35.1 Introduction

Keratoconus is characterized by progressive corneal thinning, irregular astigmatism, and loss of vision [1]. About 20% of the patients require keratoplasty for visual rehabilitation worldwide

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[1, 2]. Keratoconus exists in all ethnic groups with reported prevalence ranging from 54.5 to 230 per 100,000 [2–5]. Previous studies indicated that Asians have a high incidence of keratoconus [6–8]. Onset of the disease typically starts at puberty or early adulthood followed by variable progression [1, 4]. The diagnosis of keratoconus is made by a combination of clinical history, slit lamp examination, and corneal tomographic findings [9]. Despite rapid advancements of ophthalmic investigative technologies and imaging techniques in recent years, early diagnosis of the disease remains challenging. Furthermore, the rate of disease progression is variable. Despite its progressive nature, a few large-scale observational cohort studies have examined the natural history and rate of progression. Factors associated with a higher likelihood of progression included young age of disease onset, corneal scaring, and worse visual acuity at disease onset [10, 11]. Corneal cross-linking is able to stabilize the corneal curvature and visual function. Studies of our Hong Kong Chinese cohort of 45 keratoconus patients have shown cross-linking halts the progression [12]. The etiology of keratoconus is multifactorial, with both genetic and environmental risk factors, such as ultraviolet light exposure, eye rubbing, and consanguinity, contributing to the disease pathology [13]. Previous studies have suggested the inheritance of keratoconus development, including twin studies [14], familial aggregation studies [15], and linkage analyses [16, 17]. Genome-wide association studies (GWAS) and candidate gene association studies have identified over 150 gene polymorphisms in more than 60 genes/loci related to the risk of keratoconus. In a genome-wide association study (GWAS) that involves our Hong Kong Chinese samples, 26 loci associated with central corneal thickness (CCT) have been identified. Among them, *FOXO1* and *FNDC3B* conferred a higher risk of keratoconus [18]. An exome sequencing analysis also found a *WNT10A* variant that is associated with corneal thickness and an increased risk of keratoconus [19]. However, most of these associations were inconsistent across different study cohorts. Roles of the genes and loci remain inconclusive. In this chapter, we attempt to summarize the current knowledge of keratoconus epidemiology, pathology, and genetics, especially in the Chinese population.

35.2 Epidemiology

During the past decade, a large number of keratoconus studies have been conducted in European, American, Asian, African, and Australian populations that contribute important information that help detection and treatment (Table 35.1). Globally, the prevalence of keratoconus ranged from 54.5 to 230 per 100,000 [2–5]. Such wide range may be partly due to the nonuniform diagnostic criteria employed by different studies and partly caused by genetic variations. Hence rigorous, multiethnic, population-based epidemiological studies for keratoconus are needed. While occurrence of keratoconus is polyethnic, Asians have higher incidence, younger age of onset, and faster progression compared to other ethnicities [6–8]. Among Asians, Indians [20], Pakistanis [21, 22], Saudi Arabians [23], and Iranians (22.3–

Table 35.1 Reported incidence of keratoconus worldwide

Region	Coverage	Study period	Incidence	Source	
American	Olmsted County, Minnesota	1935–1982	54.5 per 100,000	Kennedy RH et al. 1986	[2]
Saudi Arabia	Asir Province	2001–2002	20 per 100,000	Assiri AA et al. 2005	[23]
Iranian	Yazd province	2008–2009	22.3–24.9 per 100,000	Ziaei H et al. 2012	[24]
Japanese	Tokyo	2000	12 per 100,000 (males) 5.6 per 100,000 (females)	Ota R et al. 2002	[25]
Chinese	Beijing	2011	700–1100 per 100,000	Xu L et al. 2012	[26]

24.9 per 100,000) [24] have higher prevalence and incidence. The incidence is relatively lower (12 in 100,000 males and 5.6 in 100,000 females) in the Japanese [25]. In Chinese, a population-based study calculated the prevalence of steep cornea/keratoconus of Chinese to be 0.9 ± 0.2% in the Beijing Eye Study [26]. The low prevalence may not represent the prevalence for the whole of China. More population-based epidemiological studies in different parts of China are required. According to currently reported epidemiologic data, there are indications that ultraviolet irradiation exposure, geographic locations, and environment-related ocular conditions, such as atopy, eye rubbing, and consanguinity, may be the underlying causes for the higher prevalence.

35.3 Symptoms and Diagnosis

Clinical features of keratoconus vary according to the stage of disease. There is a spectrum in physical signs: from keratoconus suspect to forme fruste keratoconus, to progressive advanced keratoconus. It is therefore difficult to determine the exact onset of disease. As the disease progresses, slit lamp should detect Fleischer's ring, Vogt's striae, and central or paracentral stromal thinning. In advanced stages, corneal scars can be evident [10]. But the patients may present with the complication of acute hydrops. In earlier stages of the disease, the cornea may appear normal on a cursory slit lamp examination, but ophthalmic investigations by retinoscopy, keratometry, and corneal topography may yield additional evidence to aid diagnosis. The increasing utilization of tomographic techniques and corneal imaging greatly assist the detection and monitor its progression. Major topographic patterns found in keratoconus include asymmetric bowtie, skewed radial axis, and inferior steepening [27]. Scheimpflug tomography, especially Pentacam® (Oculus Optikgeräte GmbH; Wetzlar, Germany), is widely applied for diagnosis of keratoconus. Various diagnostic indices derived from tomography, such as Keratoconus Severity Index (KSI) and Belin-Ambrosio Enhanced Ectasia Display (BAD), are able to discriminate normal and keratoconus corneas [28–30]. Although the diagnosis of advanced keratoconus can still be made based on the presence of abnormal corneal tomographic indices, differentiation of forme fruste keratoconus from normal corneas can be difficult [31]. Albeit an abundance of indices, there is currently no consensus on the optimal cutoff values or a single best parameter in diagnosing or monitoring keratoconus. Keratoconus corneas also have changes in biomechanical properties. Ocular Response Analyzer (ORA, Reicherts®) [32] and Corvis ST (Oculus Optikgeräte GmbH; Wetzlar, Germany) [33, 34] have been used to perform biomechanical assessment and help differentiating keratoconus from normal eyes. Combining tomographic and biomechanical data collected by the Pentacam and Corvis ST devices, we found comparable diagnostic abilities to differentiate keratoconus from normal subjects [35, 36]. The Amsler-Krumeich classification is a commonly used diagnostic tool. It combines information from biomicroscopy, keratometry, refraction, and pachymetry for keratoconus staging [37, 38]. The ultimate goal of clinicians and researchers is to improve the sensitivity of screening methods in order to accurately and reliably identify early-stage keratoconus, perform accurate classification, initiate intervention, and prevent iatrogenic keratectasia following elective laser refractive surgery.

35.4 Disease Etiology and Pathology

Keratoconus corneas tend to have reduced number and altered orientation of collagen lamellae, causing a reduction in biomechanical stability [39]. In vivo confocal images show abnormal features in all layers of the cornea, including abnormal epithelial and stromal keratocytes [40]. Reduction in keratocyte density has also been documented in keratoconic corneas [41]. Higher systemic oxidative stress was associated with keratoconus progression [42]. Matrix-degrading enzymes (MMPs), which were responsible for the degradation of the main components of

extracellular matrix and corneal membranes, were present in keratoconic corneas and involved in the pathogenesis [43, 44]. Previous studies also identified inflammatory cytokines that were associated to keratoconus [45, 46]. Proteome analysis demonstrated degenerative process in keratoconus with abnormal mitochondrial functions, increased cell death, and lipid metabolism [47]. However, no conclusive molecular mechanism has been identified, while the etiology should be complex, affected by interactive genetic and environmental factors [13]. Keratoconus has been linked to systemic disorders including Down syndrome, Ehlers-Danlos syndrome, and osteogenesis imperfect [6, 48]. However, there is no proven direct cause-and-effect relationship. Allergic diseases have been suggested to be associated with developmental keratoconus [49, 50]. Reports based on clinical observation have implicated eye rubbing as a risk factor for keratoconus [48]. Contact lens use has been implicated in the development of keratoconus but still with little evidence [51, 52]. The etiology and pathology of keratoconus are complex.

35.5 Inherited Disease-Associated Risk Factors

Keratoconus is reportedly associated with various inherited diseases. It had been proposed to be part of generalized heritable disorders, such as Ehlers-Danlos syndrome [53]. Osteogenesis imperfecta (OI) is a connective tissue inherited disorder involving genes encoding the synthesis of type 1 collagen. The ocular features in an Italian family indicated the association of OI with keratoconus [54]. Leber congenital amaurosis (LCA) is a retinal dystrophic disease which can lead to retinopathy and severe visual impairment. Keratoconus associated with LCA has been reported in patients from Pakistan [55, 56], Israel [57], and Australia [58]. One Chinese study reported that 0.86% of the 233 investigated keratoconus patients had Down syndrome [59]. However, no such associations were noted in pediatric Down syndrome populations in other studies in Asia, including Hong Kong [60], Malaysia [61], and Korea [62].

35.6 Family Aggregation and Linkage Studies

A study from the United States reported two pairs of discordant monozygotic twins for keratoconus. Some family members demonstrated corneal topographical abnormality, suggesting genetic abnormality [14]. A profile study in New Zealand that included familial aggregation analysis has identified that the keratoconus familial rate is 23.5% [15]. Linkage analysis studies have identified chromosomal loci in isolated, i.e., sporadic, keratoconus patients, including *2p24* [63], *3p14-q13* [64], *5q14.3-q21.1* [17], *13q32* [65], *16q22.3-q23.1* [66], and *20q12* [16]. However, among all these loci, no disease-causing mutation has been identified. Family history of keratoconus has been reported in about 14% of the cases [10], but the definitive role of inheritance patterns has not been determined. There are no obvious clinical differences between familial and sporadic keratoconus. As a genetic disease, keratoconus has shown weak penetrance and demonstrated significant variability of expression. While family aggregation and linkage studies for keratoconus showed that genetic factors were associated with familial inheritance, the majority of the reported keratoconus patients are sporadic.

35.7 Keratoconus Candidate Genes

35.7.1 Visual System Homeobox 1 (*VSX1*)

The visual system homeobox 1 (*VSX1*) gene is a candidate keratoconus gene with elusive pathogenetic mechanism [67, 68]. The *VSX1* Q175H mutation was shown to play a causative role in an Indian familial segregation study [69]. The shared haplotype (p.Leu268His) that was identified in five Indian keratoconus patients from two

unrelated families suggested the possibility of a founder effect which required elucidation [70]. However, *VSX1* mutations were absent in isolated keratoconus patients [71, 72]. The associations were not consistent in two case-control studies conducted in South Korea [73, 74]. A significant association between keratoconus and *VSX1* genetic alterations (p.R166W and p. H244R) was identified in Iranian patients [75].

35.7.2 Interleukin 1 Beta (*IL1B*)

Keratoconus has been considered to be a noninflammatory corneal disease. However, genetic evidence proved that chronic inflammation may exist in the pathology. Interleukin 1 beta (*IL1B*) is a mediator of keratocyte apoptosis [76, 77]. In a Japanese population, polymorphisms in interleukin 1 beta (*IL1B*) promoter region were associated with keratoconus [78]. Screening for *IL1* gene cluster mutations in a Korean cohort identified −31*C and − 511*T linkages, associated with an increased risk for keratoconus ($P = 0.012$, OR = 2.38, 95% CI = 1.116–5.046) [79].

35.7.3 Lysyl Oxidase (*LOX*)

Lysyl oxidase (*LOX*) is related to copper-dependent amine oxidase influencing the development of lysine-derived cross-links in extracellular matrix proteins, such as collagen and elastin, which play an important role in the pathogenesis of keratoconus [80]. An association study in an Iranian population identified that lysyl oxidase (*LOX*) rs1800449 risk allele A conferred risk for keratoconus [80].

35.7.4 MPDZ-NF1B

MPDZ (multiple PDZ domain crumbs cell polarity complex component) and nuclear factor I B (NF1B or NFIB) were suggested as a genetic risk of keratoconus. Sahebjada et al. [81] investigated the association between keratoconus in Australia and six CCT-associated SNPs based on GWAS results. They found two of them, rs1324183 (*MPDZ-NF1B*, chr9:13557491; $P = 0.001$; OR = 1.68) and rs9938149 (*BANP-ZNF49*, chr16:88331640; $P = 0.010$, OR = 1.47), conferred significant association with keratoconus. An association analysis between CCT-associated variants and keratoconus in a Saudi Arabian population attempted to validate eight CCT-associated SNPs. But none of the association reached statistical significance [48]. However, the result might not be conclusive due to limitation in sample size and population variation. In a Han Chinese population in northern China, ten *MPDZ-NF1B* SNPs were assessed. rs1324183 was associated with increased risk of keratoconus (OR = 3.1) [82].

35.7.5 *COL4A3*, *COL4A4*, and COL5A1

COL4A3, *COL4A4*, and *COL5A1* are related to corneal collagen structure and development during embryonic development. Genetic association of variants in these three genes in keratoconus patients were identified in European and American cohorts [83, 84]. Another study in an Iranian keratoconus cohort evaluated the possible relationship between *COL4A4* gene polymorphisms and keratoconus and revealed rs2229813 as a risk mutation [85].

35.7.6 Other Genes

The substitution c.214 + 242C > T in *IL1RN* and a novel deletion c.2558 + 149_2558 + 203del54 in *SLC4A11* were related with keratoconus in an Ecuadorian family [86]. c.2262A > C (p. Gln754His) mutation in *DOCK9*, which is expressed in corneal epithelium as activator of small G-proteins involved in intracellular signaling networks, was shown to contribute to familial keratoconus [87]. A genomic deletion within intron 2 close to the 5′ splice junction of the superoxide dismutase 1 (*SOD1*) gene was identified in familial keratoconus, suggesting *SOD1* to be a candidate keratoconus gene. *SOD1* is involved in the superoxide radical metabolism

and oxygen toxicity defense [88]. Keratoconus, epithelial basement membrane corneal dystrophy, and Fuchs' endothelial corneal dystrophy were associated with zinc finger E-box-binding homeobox 1 (*ZEB1*) gene mutation. *ZEB1* is involved in epithelial mesenchymal transition [89]. In 89 patients from a French cohort, 38 had a history of atopic dermatitis or ichthyosis vulgaris, and 5 of them were carriers of filaggrin (*FLG*) mutants [90]. Linkage analysis and genetic association indicate involvement of the calpastatin (*CAST*) gene in genetic susceptibility to keratoconus in 262 patients in 40 white keratoconus families [91]. *CAST* is involved in many aspects of cell physiology, including proliferation, apoptosis, and migration, and therefore has been proposed to be having potential involvement in the mechanisms of keratoconus development [91].

35.8 Keratoconus Genome-Wide Association Studies

Genome-wide association studies (GWAS), together with candidate gene association studies, have identified over 150 polymorphisms in more than 60 genes/loci related to keratoconus [92]. GWAS studies on keratoconus are summarized in Table 35.2. Several genes/loci were validated, such as *HGF* [93], *LOX* [94], *FOXO1* and *FNDC3B* [18], and *RAB3GAP1* [95]. A GWAS study with 933 keratoconus patients and more than 4000 controls identified variations at the *HGF* locus with keratoconus susceptibility in Caucasians [93]. The risk factor allele (rs3735520), located near the *HGF*, has strong association with keratoconus in European origin [96]. *HGF* was also significantly associated with keratoconus in an Australian cohort [97]. HGF regulates epithelial cell motility and growth during wound healing. In 2012, another study analyzed association results of the *Lysyl Oxidase* gene (*LOX)* polymorphisms from a GWAS investigation in two independent cohorts of keratoconus patients, involving 222 Caucasian patients, 687 African Americans, 3324 Caucasian controls, and 307 individuals from 70 keratoconus families. The results strongly presented genetic evidence that *LOX* variants lead to increased susceptibility to keratoconus, with meta *P* values of 2.5×10^{-7} and 4.0×10^{-5} for LOX SNPs rs2956540 and rs10519694, respectively [94]. Our previous GWAS study has identified 26 loci associated with central corneal thickness. Among them, *FOXO1 SNP rs2721051* $P = 2.7 \times 10^{-10}$ and *FNDC3B* SNP rs4894535 $P = 4.9 \times 10^{-9}$ conferred a higher risk of keratoconus [18].

A comprehensive GWAS with 222 keratoconus Caucasian patients and 3324 suggested a novel potential keratoconus locus SNP rs4954218 at *2q21.3*, containing a candidate gene *RAB3GAP1* [95]. *RAB3GAP1* expressed a catalytic subunit of GTPase-activating protein specific for the Rab3 subfamily, which participated

Table 35.2 Allelic associations of gene variations with keratoconus using cohorts from both GWAS and subsequent replication studies

No.	Studies	Study design	Country	Ethnicity	Gene and locus	Sample size		Test for HWE
						Case	Control	
1	Burdon KP et al. 2011	GWAS + validation	Australia, America	Whites	*HGF and 12 loci*	933	4164	n.r.
2	Li X et al. 2012	GWAS + validation	America	Whites	*3p26,2q21.3,19q13.3 and 12 loci*	222	3324	In HWE
3	Bykhovskaya Y et al. 2012	GWAS + validation	America	Whites	*LOX*	222	3324	In HWE
4	Lu Y et al. 2013	GWAS + validation	Australia, Northern Ireland and America	Whites	*FOXO1* and *FNDC3B*	874	6085	n.r.
5	Cuellar-Partida G et al. 2015	Exome sequencing	Australia	Whites	*WNT10A*	621	1680	n.r.

GWAS genome-wide association study, *n.r.* not reported

in normal eye development [98]. Another exome sequencing analysis in Australian patients reported a *WNT10A* variant associated with corneal thickness and an increased risk of keratoconus [19]. However, as the results across different study cohorts were inconsistent, the roles of these genes/loci are inconclusive.

35.9 Keratoconus Meta–Analysis

We have conducted a meta-analysis on 53 single-nucleotide polymorphisms (SNPs) in 28 genes/loci to find out their genetic associations with keratoconus. Among them, eight single-nucleotide polymorphisms (SNPs) in six genes/loci are associated with keratoconus in the white population. Five genes/loci were originally from genome-wide association studies, including *FOXO1* (rs2721051), *RXRA-COL5A1* (rs1536482), *FNDC3B* (rs4894535), *IMMP2L* (rs757219; rs214884), and *BANP-ZNF469* (rs9938149). The *COL4A4* gene (rs2229813; rs2228557) was identified in previous candidate gene studies [92].

35.10 Keratoconus Genetic Studies in Chinese

Reported studies on keratoconus genetics in Chinese are summarized in Table 35.3. Polymorphisms in *IL1* gene have association with risk of keratoconus in the Han Chinese population. The altered levels of *IL1* gene could induce keratocyte apoptosis preceding keratoconus [99]. Besides, polymorphisms of a candidate keratoconus gene *TGFBI* have been confirmed to be associated with keratoconus in Han Chinese [100]. *TGFBI* plays a role in tissue injury and repair that interacts with extracellular matrix protein, which may also be involved in the pathogenesis of keratoconus [101]. In a northern Han Chinese cohort of keratoconus, 10 SNPs were studied: rs4894535 (*FNDC3B*), rs3735520 (*HGF*), rs1324183 (*MPDZ-NF1B*), rs1536482 (*RXRA-COL5A1*), rs7044529 (*COL5A1*), and rs9938149 (*BANP-ZNF49*). SNP rs1324183 in *MPDZ-NF1B* was associated with an increased risk of keratoconus (OR = 3.1) [82].

Our previous GWAS identified 26 loci associated with central corneal thickness, among them, *FOXO1* and *FNDC3B* conferred a higher risk of keratoconus [18]. The candidate genes *VSX1* and *IL1A* showed genetic variations and mutations in Han Chinese keratoconus population [102]. An association analysis in Asians identified four novel loci associated with central cornea thickness in chromosomal regions *6q14.1*, *7q11.21*, *9p23*, and *15q26.3*. Rs1538138 located on chromosome 6q14.1 near the *IBTK* gene was the most significant, meta $P = 2.10 \times 10^{-11}$ [103]. Independent genome-wide association studies of corneal curvature across 10,008 Asian samples of Chinese, Malay, and Indian ancestries in Singapore identified two loci that were associated with corneal curvature variation: *FRAP1* on chromosome *1p36.2* and *PDGFRA* on chromosome *4q12* [104]. Meanwhile, mitochondrial DNA (mtDNA) is involved in mitochondrial function and affects the generation of reactive oxygen species. One study in China demonstrated that decreased integrity, content, and increased transcript level of mtDNA are associated with keratoconus [105]. Though mitochondrial haplogroups H and R were identified in Saudi Arabian keratoconus patients [106], another study in Han Chinese suggested that mtDNA copy number, but not haplogroup, is related to keratoconus [107]. In the future, large-scale multicenter genomic studies should be conducted in Chinese cohorts to establish the genetic profile of keratoconus.

35.11 Conclusive Remarks

Keratoconus is a global visual threat with complicated environmental and genetic causative factors. The progression of keratoconus can be imperceptible, and the diagnosis of keratoconus in the early stage is not easy. The pathogenesis of keratoconus is heterogeneous and complex. Epidemiological studies showed higher prevalence, earlier onset, and greater progression in Asians. Both environmental and genetic factors

Table 35.3 Allelic associations of gene variations with keratoconus in Chinese

No.	Gene/locus	SNP	Study design	Phenotype	Associated allele vs. reference allele	Sample size		Outcome		References	
						Case	Control	P	OR (95% CI)		
1	*IL1A*	rs2071376	CG	KCN	A vs. C	115	101	0.017	1.968 (1.313–3.425)	Wang Y et al. 2016	[99]
		rs2071376	CG	KCN	A vs. C	97	101	0.0487	1.51 (1.00–2.26)	Wang Y et al. 2013	[102]
2	*IL1B*	rs1143627	CG	KCN	C vs. T	115	101	<0.0001	2.846 (1.631–4.968)	Wang Y et al. 2016	[99]
		rs16944	CG	KCN	A vs. G	115	101	0.002	2.401 (1.396–4.161)	Wang Y et al. 2016	[99]
3	*MPDZ-NF1B*	rs1324183	CG	KCN	A vs. C	210	191	0.005	3.108 (1.366–7.072)	Hao XD et al. 2015	[82]
		rs1324183	GWAS + validation	CCT	A vs. C	2681		2.92 × 10–4	β: −3.83 SE:1.06	Cornes BK et al. 2012	[103]
4	*LOX*	rs2956540	CG	KCN	G vs. C	210	191	0.042	0.664 (0.447–0.986)	Hao XD et al. 2015	[82]
5	*FOXO1*	rs2721051	GWAS + validation[a]	CCT	T vs. C	874	6085	2.7 × 10–10	1.62 (1.4–1.88)	Lu Y et al. 2013	[18]
6	*FNDC3B*	rs4894535	GWAS + validation[a]	CCT	T vs. C	874	6085	4.9 × 10–9	1.47 (1.29–1.68)	Lu Y et al. 2013	[18]

7	*VSX1*	rs56157240	CG	KCN	T vs. A	97	101	0.0499	6.42 (0.77–53.78)	Wang Y et al. 2013	[102]
		rs12480307	CG	KCN	C vs. T	97	101	0.0499	6.42 (0.77–53.78)	Wang Y et al. 2013	[102]
		rs6050307	CG	KCN	T vs. G	97	101	1.22 × 10–7	0.05 (0.01–0.23)	Wang Y et al. 2013	[102]
8	*FRAP1*	rs17036350#	GWAS + validation	CC	T vs. C	4289		4.06 × 10–13	n.r.	Han S et al. 2011	[104]
9	*PDGFRA*	rs2114039#	GWAS + validation	CC	C vs. T	4289		1.33 × 10–9	n.r.	Han S et al. 2011	[104]
10	*6q14.1 near IBTK*	rs1538138	GWAS + validation	CCT	T vs. C	2681		9.49 × 10–5	β: −3.87 SE:0.99	Cornes BK et al. 2012	[103]
11	*15q26.3*	rs4965359	GWAS + validation	CCT	A vs. G	2681		1.24 × 10–4	β: −3.52 SE:0.92	Cornes BK et al. 2012	[103]
12	*7q11.21*	rs4718428	GWAS + validation	CCT	G vs. T	2681		0.0180	β: −2.31 SE:0.98	Cornes BK et al. 2012	[103]

CG candidate gene association study, *GWAS* genome-wide association study, *KCN* keratoconus, *CCT* central corneal thickness, *CC* corneal curvature. *n.r* not reported
[a]GWAS involve Chinese, replication in Caucasians #Lead SNP

play roles in the etiology and pathogenesis of keratoconus, including age, gender, ocular atopy, eye rubbing, family history, systemic inherited diseases, and genetic profiles. Family aggregation and linkage studies indicated genetic abnormality in keratoconus. GWAS and candidate gene studies identified polymorphisms in genes/loci related to the risk of keratoconus. Currently, the epidemiological and genetic data are insufficient as conclusive evidence on the role of genetic factors in keratoconus. Large, multicenter population-based studies, with age-standardized rates, random sampling, progression follow-ups, and more accurate and standardized protocol for diagnosis, are warranted in the future for better understanding of keratoconus genetics. Particularly, the limited information of keratoconus patients in China urge for further genetic studies in the Chinese population.

Compliance with Ethical Requirements Yu Meng Wang, Ka Wai Kam, Tommy C. Y. Chan, Alvin L. Young, Vishal Jhanji, Guy L. J. Chen, and Calvin C. P. Pang declare that they have no conflict of interest.

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36

Granular Corneal Dystrophy Type 2: Prevalence in South Korea, Molecular Pathogenesis, and Therapeutic Approaches

Hun Lee, Seung-il Choi, Kyung Eun Han, Tae-im Kim, and Eung Kweon Kim

Abstract

Recent advances in the genetics of corneal dystrophies have facilitated more precise classification of the disease and elucidation of the molecular mechanisms involved in the pathogenesis of the different types of corneal dystrophies. Granular corneal dystrophy type 2 (GCD2) is an autosomal dominant disorder associated with the arginine-to-histidine substitution at codon 124 (R124H) in the transforming growth factor-β-induced gene (TGFBI) on chromosome 5q31. The hallmarks of GCD2 include the age-dependent progressive accumulation of transforming growth factor-β-induced gene protein (TGFBIp) in the corneal stroma in the form of hyaline and amyloid, which interfere with corneal transparency. The heterozygous form of GCD2 is generally mild when the patients are young, accompanied by only a few visually insignificant corneal opacities. However, as patients age, increased diffuse haze will appear, resulting in decreased vision owing to reduced passage of light rays through the visual axis. In contrast, patients with the homozygous form of GCD2 have severe visual impairment beginning early in childhood. Here, we discuss the current state of knowledge on GCD2, including the epidemiology, clinical manifestations, molecular pathogenesis, and treatment modalities.

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H. Lee
Department of Ophthalmology, International St. Mary's Hospital, Catholic Kwandong University College of Medicine, Incheon, South Korea

Department of Ophthalmology, Vision Research Institute, Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea

S.-i. Choi
Corneal Dystrophy Research Institute, Yonsei University College of Medicine, Seoul, South Korea

K. E. Han
Department of Ophthalmology, Ewha Womans University, Seoul, South Korea

T.-i. Kim
Department of Ophthalmology, Vision Research Institute, Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea

Corneal Dystrophy Research Institute, Yonsei University College of Medicine, Seoul, South Korea

E. K. Kim (✉)
Department of Ophthalmology, Vision Research Institute, Severance Hospital, Yonsei University College of Medicine, Seoul, South Korea

Corneal Dystrophy Research Institute, Yonsei University College of Medicine, Seoul, South Korea

Severance Biomedical Science Institute, Yonsei University College of Medicine, Seoul, South Korea
e-mail: eungkkim@yuhs.ac

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Keywords

Granular corneal dystrophy type 2 · Prevalence · Molecular mechanism · Genetics · South Korea

36.1 Introduction

Recent advances in the genetics of corneal dystrophies have facilitated more precise classification of the disease and elucidation of the molecular mechanisms involved in the pathogenesis of the different types of corneal dystrophies. The International Committee for Classification of Corneal Dystrophies (IC3D) provides updated data to ophthalmologists by incorporating traditional definitions of corneal dystrophies with new genetic, clinical, and pathologic information. Granular corneal dystrophy type 2 (GCD2) is an autosomal dominant disorder associated with the arginine-to-histidine substitution at codon 124 (R124H) in the transforming growth factor-β-induced gene (*TGFBI*) on chromosome 5q31 [1]. The hallmarks of GCD2 include the age-dependent progressive accumulation of transforming growth factor-β-induced gene protein (TGFBIp) in the corneal stroma in the form of hyaline and amyloid, which interfere with corneal transparency [2, 3]. The heterozygous form of GCD2 is generally mild when the patients are young, accompanied by only a few visually insignificant corneal opacities. However, as patients age, increased diffuse haze will appear, resulting in decreased vision owing to reduced passage of light rays through the visual axis. In contrast, patients with the homozygous form of GCD2 have severe visual impairment beginning early in childhood. However, the exact molecular mechanisms mediating the pathogenesis of GCD2 are not fully understood.

In this chapter, we discuss the current state of knowledge on GCD2, including the clinical manifestations, epidemiology, molecular mechanisms, and treatment modalities.

36.2 Epidemiology of GCD2

Direct measurement of a population-based sample to determine the prevalence of GCD2 would be prohibitively expensive owing to the relatively low prevalence of GCD2 in most countries. Our group investigated the prevalence of GCD2 in the Korean population by identifying the number of patients with the homozygous form of GCD2 as a measure of the frequency of heterozygous GCD2 [4]. We found 16 families, including 21 children, who were homozygous for the R124H mutation. All parents of homozygous patients available for examination in the study were themselves heterozygous for the R124H mutation. Thus, with the assumption of the Hardy-Weinberg equilibrium, we used the variable q for the prevalence of the GCD2 allele (A') in the Korean population and the variable p for the prevalence of the normal allele (A). The prevalence of the normal allele (A) is $(1 - q)$, and for convenience, we let the variable $p = (1 - q)$. The Hardy-Weinberg equation says that the genotype frequencies for the normal allele (AA), heterozygous allele (AA'), and homozygous allele (A'A') are given by p^2, $2pq$, and q^2, respectively [4], and the sum of the prevalence for normal, heterozygous, and homozygous alleles must be exactly one ($p^2 + 2pq + q^2 = 1$). Using the Korean registration-based census of 47,041,434 individuals, the estimated overall prevalence (combining heterozygotes and homozygotes) of GCD2 is 11.5 per 10,000 persons ($q = 0.000574$) based upon the Hardy-Weinberg principle. In another study conducted in South Korea, GCD2 was the most frequent mutation among 268 patients with *TGFBI* corneal dystrophies [5].

36.3 Asian Perspective

According to the results of several studies, substitution of arginine for histidine at codon 124 (R124H) is considered to be the most frequently observed mutation in the Asian population. In China, granular corneal dystrophy type 1 (GCD1) is the most popular mutation, followed by lattice

corneal dystrophy type 1 (LCD1) and GCD2 [6]. According to Japanese studies, R124H mutation is the most common mutation, up to 72% of patients with corneal dystrophies [7, 8]. The second most frequently observed mutation is either LCD1 or GCD1. In Western countries, LCD1 is more common. Notably, the prevalence of *TGFBI* corneal dystrophies is likely to be underestimated as these data were collected only from published studies.

36.4 Etiology and Clinical Features

Substitution of arginine for histidine at codon 124 (R124H) in the transforming growth factor-β-induced gene (*TGFBI*) on chromosome 5q31 is always found in GCD2 [1]. Age-dependent progressive accumulation of hyaline and amyloid and the production of TGFBIp, an extracellular matrix protein, in the corneal epithelia and stroma are also generally observed in GCD2 [2, 3]. Most TGFBIp (also known as keratoepithelin) expressed under normal conditions is thought to be produced by the corneal epithelium. During wound healing in the normal human cornea, TGFBIp is found in the epithelium and fibroblasts near the wound, which suggests that keratocytes can also produce TGFBIp [9]. TGFBIp can be deposited in corneal tissue in the three major forms of dominant *TGFBI*-linked corneal dystrophies, i.e., the granular form (GCD types 1 and 2), lattice form (LCD types 1, 3, and 4), and diffuse Bowman's layer deposits (Reis-Bucklers and Thiel-Behnke corneal dystrophies) [2].

GCD2 exhibits the clinical and histological characteristics of both granular and lattice dystrophies. Clinically, small and faint superficial stromal deposits observed in the first or second decade of life with a slit lamp are the earliest manifestations (Fig. 36.1) [1, 10]. The heterozygous form of GCD2 is generally mild, and visually insignificant corneal opacities appear at a young age. These granular deposits accumulate with age. In particular, lattice-like amyloid lesions appear in some patients in the deeper stroma with superficial granular lesions, and a superficial diffuse anterior stromal haze appears in some older patients (Fig. 36.2). Coalescence of the opacities may impair visual acuity in elderly patients with the heterozygous form of the disease. However, the homozygous form of GCD2 has earlier onset with faster progression showing many gray-white, discrete deposits in the superficial cornea compared with the heterozygous form of GCD2 [11–13]. Specifically, these deposits can be observed as early as 3 years of age in patients with the homozygous form of GCD2 and increase in size with age (Fig. 36.3). Thus, patients with the homozygous form of GCD2 have severe visual impairment from early in childhood, eventually requiring surgery. Our group reported that some granular corneal

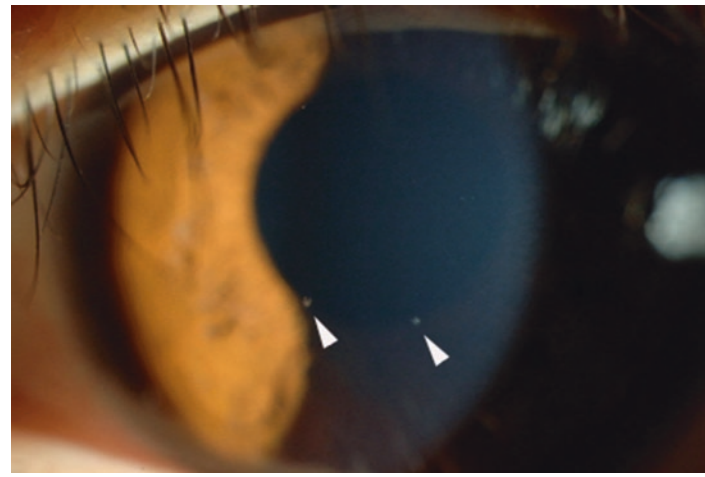

Fig. 36.1 Slit-lamp photograph of a 23-year-old female patient with heterozygous granular corneal dystrophy type 2. Two small granular deposits are present in the anterior stroma

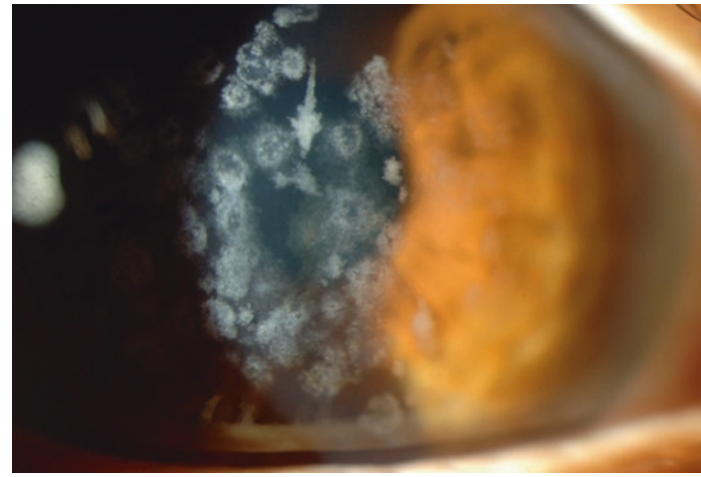

Fig. 36.2 Slit-lamp photograph of a 69-year-old female patient with heterozygous granular corneal dystrophy type 2. Multiple crumb-shaped granular deposits are present in the anterior stroma, and deep lattice-like deposits are present in the deep stroma. A diffuse haze is present between granules

Fig. 36.3 Slit-lamp photograph of a 6-year-old male patient with homozygous granular corneal dystrophy type 2. Multiple crumb-shaped granular deposits are present in the anterior stroma

deposits disappear partially after painful corneal erosion attack [14]. The dropping out of the center of deposits would result in the formation of ring-shaped opacities [14]. Histologically, granular deposits in the anterior stroma stain with Masson's trichrome, while the lattice-like lesions in the deep stroma stain with Congo red. Electron microscopy has revealed the presence of rod-shaped or trapezoidal electron-dense areas surrounded by tubular microfibrils [15].

Notably, in patients with the heterozygous form of the disease, manifestation of the damage to the cornea is exacerbated when LASIK or LASEK procedures are applied (Figs. 36.4 and 36.5). Because the prevalence of the heterozygous form of GCD2 in Korea is 1 in 870, more than 300 patients have experienced poor vision after undergoing LASIK or LASEK procedures in Korea.

36.5 Molecular Pathogenesis of GCD2

The pathogenesis of GCD2 has been shown to be associated with altered morphological characteristics of corneal fibroblasts, vulnerability of corneal fibroblasts to oxidative stress, mitochondrial dysfunction, defective autophagy, and delayed mutant-TGFBIp secretion.

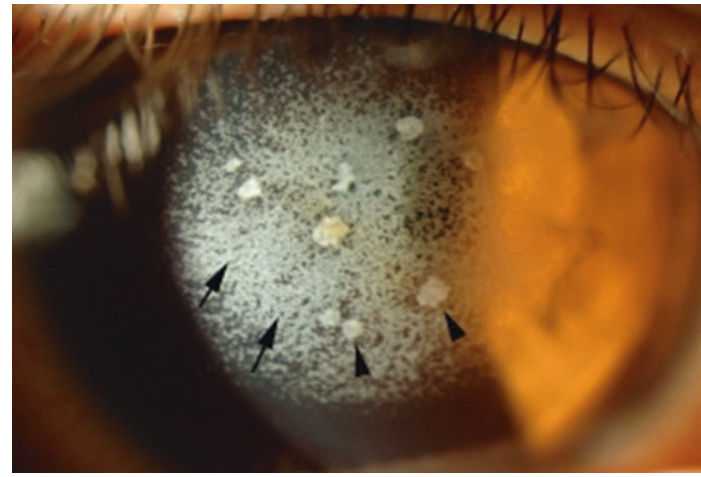

Fig. 36.4 Slit-lamp photograph of a 28-year-old male patient with heterozygous granular corneal dystrophy type 2 at 6 years after LASIK. Newly formed small granules (arrows) are present along the LASIK flap interface with multiple crumb-shaped granular deposits (arrow heads) in the anterior stroma

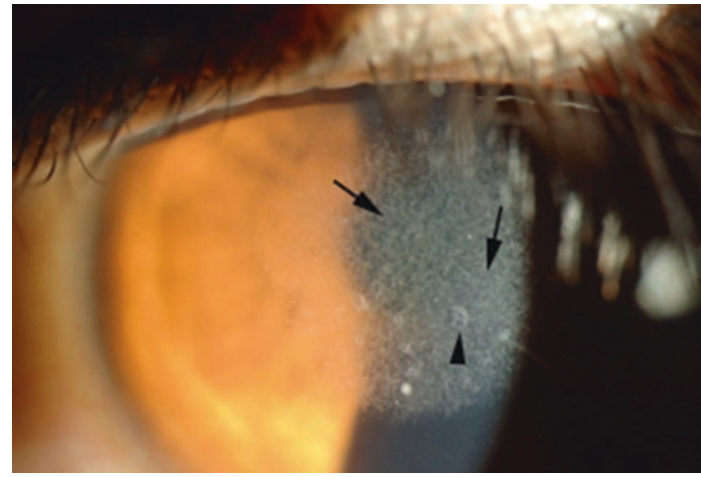

Fig. 36.5 Slit-lamp photograph of a 25-year-old female patient with heterozygous granular corneal dystrophy type 2 after LASEK. Newly formed small granules (arrows) are present with a crumb-shaped granular deposit (arrow head) in the anterior stroma

36.5.1 Morphological Properties of GCD2 Corneal Fibroblasts

Keratocytes (often called corneal fibroblasts when cultured) are the predominant cellular component of the corneal stroma and are mainly involved in maintaining corneal transparency and the extracellular matrix environment [16]. In eyes with GCD2, corneal fibroblasts harbor multiple vesicles of different sizes containing amorphous material showing a senescence-like morphology with increases in size [17, 18]. Additionally, cultured GCD2 corneal fibroblasts exhibit a much more extensive pattern of colocalization for mutant-TGFBIp and cathepsin D (an enzyme found in lysosome) than wild-type cells [17]. Transmission electron microscopy analyses have revealed that GCD2 corneal fibroblasts contain fragmented or elongated mitochondria, whereas normal corneal fibroblasts contain small, round mitochondria [19, 20]. Disorganized and dilated mitochondria are often observed, particularly in GCD2 homozygous corneal fibroblasts at late passages. Corneal fibroblasts from patients with the homozygous form of GCD2 do not survive for more than eight passages in vitro, whereas those from normal donors can survive for more than 24 passages.

36.5.2 Oxidative Stress in GCD2 Corneal Fibroblasts

In mammalian cells, aerobic metabolic processes lead to the production of reactive oxygen species (ROS) in mitochondria and peroxisomes. Excessive ROS can cause oxidative damage to proteins, lipids, and DNA. Because the cornea should be transparent in order to *allow light transmission*, it should be avascular. The cornea is also constantly exposed to a wide spectrum of light, including ultraviolet (UV) light, which causes tissue stress and ROS generation [21]. These factors make the cornea more vulnerable to oxidative stress than other tissues. Accordingly, the cornea is rich in antioxidant enzyme systems that aid in the removal of ROS generated by UV light absorption [22]. Oxygen is reduced to water (H_2O) by the passage of electrons through electron transport chain redox reactions coupled with proton transfer across the inner mitochondrial membrane in the mitochondria. Superoxide ($O_2^{\bullet -}$), hydroxyl radicals ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) are formed as a side effect of this process [23]. Excessive production of O_2^- and H_2O_2 results in *oxidative injury* to the *tissue*. To maintain a healthy status, many types of mammalian cells have developed both enzymatic and nonenzymatic ROS scavenging mechanisms. When the redox-active species metabolic system is unbalanced, more oxidizing agents are produced, and oxidative stress occurs. Indeed, increased amounts of malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), protein carbonyl groups, and 8-hydroxy-2′-deoxyguanosine (8-OHdG) are frequently associated with oxidative damage (Fig. 36.6) [24–29].

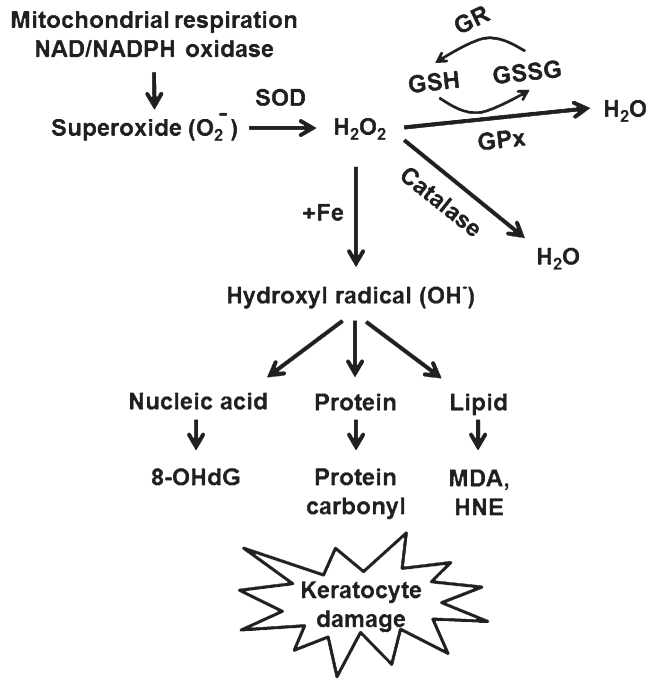

Fig. 36.6 Oxidative stress, antioxidant defenses system, and keratocyte damage. A primary source of reactive oxygen species (ROS) production is mitochondrial NADPH/NADH oxidase. Hydrogen peroxide (H_2O_2) is converted to the hydroxyl radical ($^{\bullet}OH$) in the presence of transition metals, such as iron (+Fe). The hydroxyl radical induces DNA, protein, and lipid damage, leading to cell damage and release of 8-OHdG, protein carbonyl group, malondialdehyde (MDA), and 4-hydroxynonenal (HNE) components. Cells have protective antioxidant systems, including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione (GSH). Upon oxidation, GSH forms glutathione disulfide (GSSG)

Our group examined the mitochondrial features of heterozygous GCD2 corneas using electron microscopy and demonstrated that GCD2 corneas have many mitochondria that are dilated and degenerative and/or contain vesicles with amorphous material [30]. MitoTracker and cytochrome *c* staining showed increased mitochondrial activity in mutated cells in early passages in homozygous GCD2 corneal fibroblasts cultured for four to eight passages. In late-passage mutant cells, decreases in depolarized mitochondria, cellular proliferation, and complexes I–V expression were observed. Treatment of the cultured cells with butylated hydroxyanisole, an antioxidant, was shown to result in decreased intracellular ROS and reduced mitochondrial oxidative damage [30].

Our group also demonstrated that oxidative damage is involved in GCD2 pathogenesis by showing that levels of 4-HNE, MDA, and protein carbonyl groups were significantly elevated in cultured GCD2 corneal fibroblasts compared with those in normal corneal fibroblasts [31]. We also examined MDA distribution in the corneal tissues from patients with GCD2 by immunohistochemical staining and demonstrated that MDA expression was significantly elevated in the corneal stroma and epithelium from patients with GCD2; notably, however, MDA immunoreactivity was also detectable in corneal epithelial cells from age-matched normal controls [31]. Moreover, the expression levels of Cu/Zn-superoxide dismutase (SOD), Mn-SOD, glutathione peroxidase (GPx), and glutathione reductase (GR) proteins in GCD2 corneal fibroblasts are significantly elevated compared with those in normal corneal fibroblasts. Intracellular ROS and H_2O_2 levels are significantly elevated in GCD2 corneal fibroblasts compared with those in wild-type corneal fibroblasts [31].

36.5.3 Accumulation and Degradation of TGFBIp via Autophagy Pathway in GCD2 Corneal Fibroblasts

Many degenerative disorders are characterized by the accumulation of intracellular or extracellular protein aggregates, which eventually result in cellular death. To control the quality of intracellular proteins and organelles, cells have special surveillance systems. Two major systems, the ubiquitin/proteasome system (UPS) and the autophagy system, are responsible for the proteolysis and removal of abnormal proteins [32]. Several studies have shown that the accumulation of disease-related proteins is highly dependent on the UPS and/or the autophagy system [33, 34]. Degradation of proteins by the UPS is also known to be a regulated, specific process [35]. Degradation of a target protein by the UPS occurs in two successive steps: (1) several ubiquitin molecules are conjugated to the target protein, a process that is catalyzed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and a substrate-specific ubiquitin-protein ligase (E3); and (2) the tagged protein is degraded into small peptides by the 26S proteasome complex, which recognizes only ubiquitin-conjugated proteins [36, 37].

Autophagy occurs by sequestering molecules and organelles for degradation by lysosomes. Autophagy can be classified into the following three types: microautophagy, chaperone-mediated autophagy, and macroautophagy [38]. Hereafter, we refer to the process of macroautophagy as autophagy. In the autophagy pathway, bulky cytoplasmic materials and organelles are packaged into double-membrane-bound vesicles called autophagosomes. These autophagosomes then fuse with lysosomes, and damaged material is degraded within these fused vesicles through the functions of lysosomal enzymes (Fig. 36.7) [39].

In the context of CD2, mutant TGFBIp has been shown to accumulate in the autophagolysosomal compartment of corneal fibroblasts [17, 18]. In GCD2 corneal fibroblasts, mutant-TGFBIp colocalizes extensively with microtubule-associated protein 1 light chain 3b (LC3)-enriched cytosolic vesicles, cathepsin D, and lysosomal enzymes, indicating that TGFBIp is degraded by autophagy [17, 18]. Furthermore, when GCD2 corneal fibroblasts are treated with bafilomycin A1, an inhibitor of the fusion of autophagosomes to lysosomes, caspase-3 and

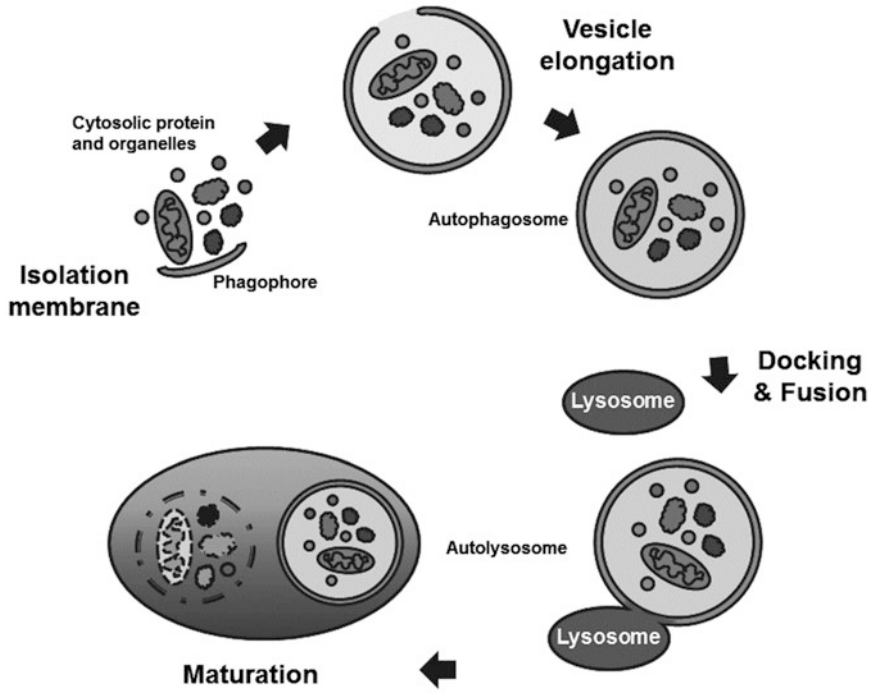

Fig. 36.7 The autophagy process. In the presence of an autophagy inducer, cytosolic proteins and organelles are sequestered by a pre-autophagosomal membrane structure called the phagophore. The phagophore membrane then expands and encloses its cargo to form a double-membrane vesicle, the autophagosome. The outer membrane of the autophagosome docks and directly fuses with a lysosome to form an autolysosome. The sequestered material is degraded inside the autolysosome and recycled later

poly (ADP-ribose) polymerase 1 (PARP1) are activated [17]. Based on these results, defective autophagy is thought to contribute to GCD2 corneal fibroblast dysfunction.

Hence, induction of autophagy may be an option for GCD2 treatment even though autophagy itself is not sufficient for the removal of all mutant-TGFBIp from GCD2 corneal fibroblasts [18]. When rapamycin, an autophagy inducer, is applied to cultured GCD2 corneal fibroblasts, mutant-TGFBIp levels are reduced. However, normal TGFBIp levels in wild-type corneal fibroblasts did not change [17]. This suggests that rapamycin or its related analogs may be suitable agents for the treatment of patients with GCD2. Because rapamycin is currently used in the clinical setting to treat several diseases, application of rapamycin in patients with GCD2 may be a promising strategy [17, 40]. Our group also demonstrated that melatonin functions as an inducer of autophagy via a mammalian target of rapamycin (mTOR)-dependent pathway and consequently eliminates mutant-TGFBIp from GCD2 corneal fibroblasts (Table 36.1) [41]. When melatonin and rapamycin are applied together, they have greater effects on mutant-TGFBIp clearance than either drug alone.

Our group also showed that mitomycin C (MMC) application during photorefractive keratectomy (PRK) to GCD2 corneas did not inhibit the exacerbation of GCD2 after laser ablation [42]. When MMC was applied to cultured fibroblasts harvested from normal, heterozygous GCD2 and homozygous GCD2 cornea, MMC caused apoptosis in GCD2 corneal fibroblasts, with homozygous cells being most vulnerable. Because TGFBIp can be absorbed from outside of corneal fibroblasts into the cell and can be degraded in lysosomes, application of MMC to GCD2 corneas to inhibit the formation of deposits

Table 36.1 Effects of melatonin on TGFBIp, autophagy, and mTOR signaling pathway

Parameters	Wild type	Granular corneal dystrophy type 2
LC3 I	+	+
LC3 II	+	+
Autophagosome	+	n.t.
ATG5	+	n.t.
mTOR	n.c.	n.t.
p-mTOR (2481)	n.c.	n.t.
p-mTOR (2448)	+	+
GβL	n.c.	n.t.
Raptor	n.c.	n.t.
Rictor	n.c.	n.t.
TGFBIp	–	–

-, decrease; +, increase; n.t., not tested; n.c., not changed significantly; LC3, microtubule-associated protein 1 *light chain 3*; ATG5, autophagy-related gene 5; mTOR, mammalian target of rapamycin; GβL, G-protein-β-subunit-like protein; Raptor, regulatory-associated protein of mTOR; Rictor, rapamycin-insensitive companion of mTOR; TGFBIp, transforming growth factor-β-induced protein

is not desirable [43]. Further studies are needed to fully elucidate the roles of keratocytes in the pathogenesis of GCD2.

36.5.4 TGFBIp Regulation via TGF-β Signaling Pathway: Therapeutic Applications

The transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates diverse cellular and physiological processes, including proliferation, differentiation, and extracellular matrix homeostasis [44]. This pathway is initiated by TGF-β binding to type I (TβRI) and type II (TβRII) TGF-β receptors, both of which can activate multiple downstream signaling pathways to alter gene transcription (Fig. 36.8). The activated receptor complex phosphorylates the downstream transcription factors Smad2 and Smad3, promot-

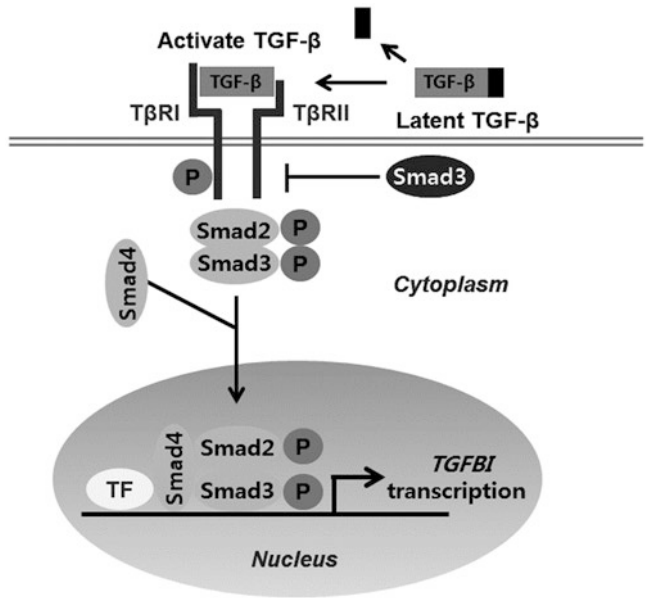

Fig. 36.8 Major transforming growth factor-β signaling pathways. Latent TGF-β complexes are activated. Activated TGF-β then assembles the productive signaling heteromeric receptor complex. In the signaling receptor complex, the type II receptor (TβRII) activates its intracellular serine/threonine kinase function, resulting in multiple intracellular signaling cascades. Thus, once TGF-β1 binds to this receptor complex, intracellular Smad2 and Smad3 are recruited and phosphorylated at their C-termini by TβRI. Phosphorylated Smad2 and Smad3 subsequently bind to Smad4 and translocate to the nucleus, where they modulate the transcription of target genes in collaboration with cofactors and other transcription factors (TFs)

Table 36.2 Effects of lithium on TGFBIp, autophagy, cellular proliferation, and TGF-β signaling

Parameters	Wild type	Granular corneal dystrophy type 2
TGFBIp	–	–
LC3 I	n.c.	n.t.
LC3 II	+	n.t.
Smad3	–	n.t.
p-Smad3 (S423/425)	n.c.	n.t.
GSK3α/β	n.c.	n.t.
p-GSK3α/β (S21/9)	+	n.t.
Cell proliferation	n.c.	n.c.

-, decrease; +, increase; n.t., not tested; n.c., not changed significantly; LC3, microtubule-associated protein 1 *light chain 3*; GSK3, glycogen synthase kinase 3

ing their association with Smad4. The Smad complex then translocates to the nucleus to regulate the transcription of target genes (Fig. 36.8) [45]. Because TGFBIp expression is induced by TGF-β, reducing intracellular TGFBIp by controlling TGF-β signaling may be a useful approach for drug development in *TGFBI*-linked corneal dystrophies [46]. Our group reported that lithium treatment of corneal fibroblasts reduces the phosphorylation of Smad3 (S423/425) and the expression of normal and mutant TGFBIp, suggesting that lithium may be a potential useful compound in the treatment of *TGFBI*-linked corneal dystrophies (Table 36.2) [46].

36.6 Treatment Modalities

36.6.1 Laser Ablation

Phototherapeutic keratectomy (PTK) has been used for the removal of shallow corneal deposits, improving vision and delaying the need for keratoplasty. Dystrophic deposits, however, typically recur after PTK in patients with *TGFBI* corneal dystrophies. Seitz et al. concluded that PTK can increase visual acuity without affecting the prognosis of subsequent penetrating keratoplasty (PKP) [47].

36.6.2 Keratoplasty

Anterior lamellar keratoplasty (ALKP) and PKP are traditionally used to treat deep deposits of stromal corneal dystrophies. ALKP may be considered for primary cases of GCD2 or cases of recurrence following PTK for preservation of the deep corneal stroma. Visual acuity after ALKP, however, is often less than that after DALK due to irregularities and/or scarring on the donor-to-recipient interface [48, 49]. Several research groups have performed homologous penetrating central limbokeratoplasty; however, the success rate of this procedure has not been shown to be satisfactory because donor epithelial cells are eventually replaced by host epithelial cells [50–53].

36.7 Conclusion

In summary, we presented recent literature describing the genetics of GCD2 in Asia and the pathophysiology of GCD2. Lithium and melatonin have been proposed as possible therapeutic agents for the management of GCD2. Until now, surgical treatments, including PTK, ALKP, DLKP, and PKP, have been used to maintain adequate vision in patients with GCD2. More studies of the prevalence of GCD2, both in Asia and around the world, as well as continuous development of therapeutic agents are required.

Conflict of Interest Eung Kweon Kim is in the medical advisory board member of Avellino Lab USA. Hun Lee, Seung-il Choi, Kyung Eun Han, and Tae-im Kim declare that they have no conflict of interest.

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About the Editors

Dr. Gyan Prakash is the founding President of Asian Eye Genetics Consortium (AEGC), now renamed as Global Eye Genetics Consortium (GEGC). He has a deep-rooted dedication and passion for global health programs, international research collaborations, and more than 30 years of experience in international health research, teaching, and mentorship. Dr. Prakash has worked across many biomedical disciplines including infectious diseases, drug development, eye diseases, substance and drug abuse, oncology, neurology, and biomedical technology.

Dr. Prakash has served as Director of the Office of International Program Activities (OIPA) at National Eye Institute in the USA since 2012, and worked across interdisciplinary and geographic boundaries to lead strategic programs and opportunities for advancing scientific research, training, developing next generation of biomedical workforce, and promoting global engagement of researchers at all career stages. Earlier in his career, he worked with the industry leaders to create three new companies (Mesa Diagnostics in Albuquerque, New Mexico, Metastatin Pharmaceuticals in Bethesda, Maryland, and AMAR International in Reston, Virginia), led teams at established companies (Pfizer International in New York, Johnson and Johnson/Ortho Diagnostics in California, and PRA International in Virginia), and mentored the next generation of global health scientists and R & D managers at NIH-Global Health Interest Group (NIH-GHIG), George Mason University, University of Maryland, and Johns Hopkins University.

Dr. Prakash created and established over a dozen innovative and fully operational product development programs in the pharmaceutical and biotechnology industries. He served as the Project Scientist for Pfizer's first therapeutic biologic (first therapeutic monoclonal Antibody – E5), and then for fluconazole, the number one selling antifungal drug in the world. Dr. Prakash earned his first international program management experience working at the Pfizer world headquarters in New York in many leadership roles over several years. At Pfizer, he administered thirty-four international clinical programs across the globe for the largest selling antifungal drugs in the world, fluconazole and voriconazole, which have been used by millions of HIV/AIDS and cancer patients and saved millions of lives around the world in the immunosuppressed and immunocompromised patient populations. While at Pfizer and then at NIH-NIAID, he worked with the clinical investigators and scientists in low and middle income countries on various programs related to infectious and neglected tropical diseases such as TB, malaria, influenza, and visceral leishmaniasis. His experience in non-communicable diseases spans from eye diseases, cancer, and drug and substance abuse. Before joining the National Institutes of Health, he served as the Chief Operating Officer and Senior Fellow at AMAR International, Inc., a life science program management company in Reston, Virginia, USA, where he led biomedical program management teams for the US Government for large programs. Previously, Dr. Prakash served as

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the President and CEO of Metastatin Pharmaceuticals, Inc., a cancer biologics-development stage company in Bethesda, Maryland, USA.

Dr. Prakash has served in the capacity of senior scientific advisor for several public and private pharmaceutical/biotech companies, as Director for the programs of American Society for Microbiology, and a member on national committee for a major medical and professional association. Dr. Prakash held an Adjunct Professorship at Robert H. Smith School of Management, University of Maryland and served on the advisory board of New Market Growth Fund of Dingman Center for Entrepreneurship. In 2001, he was appointed an Adjunct Professor at School of Management at George Mason University in Virginia where he established the first graduate program on bioscience management in the USA. Dr. Prakash has published several original papers in peer-reviewed journals and has a major biotechnology book, Nucleic Acid and Monoclonal Antibody Probes, to his credit that he published with Marcel Decker of New York. He has also coauthored a manual of business of biotechnology and has presented numerous invited lectures around the world.

Dr. Prakash earned his graduate degrees, M.S., and Ph.D. in Medical Microbiology (University of Illinois at Urbana-Champaign, USA) and an MBA in Pharmaceutical Management/Marketing (St. Joseph's University, USA). He received professional training at the UCLA School of Management and Centers for Disease Control (CDC) in Atlanta. Prior to immigrating to the USA in 1978, Dr. Prakash earned a B.Sc. (Biology and Chemistry) and a M.Sc. (Biochemistry) at University of Allahabad in India. He received the University Gold Medal in the University for securing the highest rank in the undergraduate program. Dr. Prakash has received a number of national and international awards and recognitions in his career including a UNESCO Fellowship, AAAS award, the White House Blue Ribbon Award, Dr. Brahma Prakash Memorial Oration Award, and the Aditya Jyot Foundation Award. He has served as invited speaker in many parts of the world, such as Japan, India, China, Brazil, Australia, the UK, Spain, France, Germany, and many other countries. Dr. Prakash has been affiliated with several leading universities in the USA and abroad (Johns Hopkins, University of Maryland, Georgetown University, George Mason University, Cambridge University in the UK, and Sun-yat Sen University in China) as Adjunct and visiting Professor in teaching role and serving on various committees. He played a key role in founding of a new trans-NIH Global Health Interest Group (NIH-GHIG) in 2012, providing infrastructure and mentorship to post-doctoral fellows and next generation of scientists across NIH interested in global health. Dr. Prakash has been the founder of a nationally recognized program "Science for the Future" that won the Blue Ribbon Award from The White House. The program provided guidance in developing hands-on science as part of the early learning in elementary schools and was featured on "The Frontline" at the Public Broad-casting Service (PBS).

Dr. Takeshi Iwata received his Ph.D. from the Department of Agriculture at Meijo University in Japan and moved to the National Eye Institute (NEI)/National Institute of Health (NIH), USA, as postdoctoral fellow in a retina genetic laboratory, headed by Dr. George Inana. The laboratory had just identified the first gene responsible for genetic eye disease called gyrate atrophy caused by a mutation in ornithine aminotransferase (OAT) gene. Dr. Iwata's first job was to use this OAT cDNA probe to hybridize OAT pseudogenes in X-chromosome and perform link- age analysis for X-linked retinitis pigmentosa families. He moved with Dr. Inana to Bascom Palmer Eye Institute, University of Miami School of Medicine in Miami, Florida, to further work on other hereditary retinal diseases. After 2 years, he returned to the NEI laboratory headed by Dr. Deborah Carper to work on another major retinal disease, the diabetic retinopathy. His work was to identify the mechanism of transcrip- tional regulation for two genes, aldose reductase and sorbitol dehydrogenase, in polyol pathway associated with the disease. These retinal and gene related work in the USA motivated him to significantly

expand the research when he returned to Tokyo, Japan, to head the first laboratory at the National Institute of Sensory Organs (NISO), Tokyo Medical Center, National Hospital Organization (NHO). The NHO is a group of 143 National Hospitals with 52,000 beds and more than 59,000 total employees. Ten Research Centers in Japan are affiliated to the NHO and located in major cities with each center targeting specific medical research area. The NISO was developed as the first sensory organs research institution in Japan to focus on vision, hearing, and vocalization research.

Dr. Iwata is currently running three main projects related to age-related macular degeneration (AMD), normal tension glaucoma (NTG), and hereditary retinal diseases as the Director of the Molecular and Cellular Biology Division. His research goes beyond identification of disease-causing gene to explore the mutant biological behavior in vitro and in vivo. His laboratory recently identified the abnormal transcriptional regulation of HTRA1 gene at the most highly associated genome region on chromosome 10 (Iejima et al., J Biol Chem 2014). This hypothesis was confirmed when HTRA1 was overexpressed in mouse which led to the development of choroidal neovascularization (Nakayama et al., Invest Ophthalmol Vis Sci 2015). His research for normal tension glaucoma focuses on optineurin (OPTN) gene responsible for hereditary NTG. His laboratory identified the OPTN E50K mutant protein that interacts with TANK-binding protein 1 (TBK1), which leads to the precipitation of OPTN in the endoplasmic reticulum (Minegishi et al., Hum Mol Genet 2013). When inhibitor chemical for TBK1 was applied, this precipitation was significantly reduced. The work has now expanded to the identification of FDA approved TBK1 inhibitor drug for worldwide clinical trial (Minegishi, Nakayama, Iejima et al., Prog Retin Eye Res 2016). In 2011, Dr. Iwata started with six Japanese ophthalmologists from different universities specialized in retinal electrophysiology to systematically analyze the genetics of family with hereditary retinal diseases. This small group has now expanded to 26 universities and institutions as the Japan Eye Genetics Consortium (JEGC) for Hereditary Retinal Diseases. A diagnostic system and a database developed and described in Chap. 2 has inspired him to help establish the Asian Eye Genetics (AEGC, http://asianeyegenetics.org) using the same system. He now serves as the president of AEGC.

Dr. Iwata has received awards from Cooperative Cataract Research Group, National Foundation for Longevity Science, Foundation Preventing Blindness, Japanese Association for Complement Research, Japan Retinitis Pigmentosa Society, International Society for Eye Research (ISER) Special Recognition Award, and has given keynote lectures around the globe including major universities in the USA, UK, China, India, and recently at RD 2016 meeting in Kyoto, Japan. Dr. Iwata has served as a committee member in number of local and international organizations including the Association for Research in Vision and Ophthalmology (ARVO) and the International Society for Eye Research (ISER). He has served as a com- mittee member of the ARVO Foundation Award Committee, ARVO Global Research Training Committee, and ARVO Executive Committee Member for the Advocacy Pillar. He has served two terms as a counselor for ISER and recently served as the vice president (Asia & Pacific) and program chair for the XXII Biennial Meeting of ISER 2016 in Tokyo, Japan. Dr. Iwata has over 100 publications in scientific journals, reviews, and book chapters. He currently serves on the editorial board for *Journal of Ocular Biology, Diseases, and Informatics* and *Eye and Brain*.

Dr. Iwata has supported international collaborations between researchers, laboratories, institutions, and consortia. Recently, he played a major role in establishing several Collaborative Research Agreements including the ones between NISO and NEI=NIH in the USA, Buffalo Niagara Medical Center Campus in the USA, Aditya Jyot Eye Hospital, Mumbai in India, and Moorfield Eye Hospital-University College, London in the UK. These collaborations have brought fruitful results to NISO-Tokyo Medical Center. As the President of AEGC (now GEGC), Dr. Iwata is actively

involved in identifying the key leaders around the world to build effective plans for future genetic eye research. He led the third annual meeting of AEGC (now GEGC) during ARVO 2016 in Seattle with over 45 attendants representing each region of Asia. The meeting participants agreed to share eye genetic information by constructing a common AEGC (now GEGC) database containing useful genotype-phenotype information along with natural history of patients for each mutation recorded. AEGC (now GEGC) database for Stargardt's Disease and Occult Macular Dystrophy started operation in September, 2016, initiated by Dr. Kaoru Fujinami. These AEGC (now GEGC) database should accelerate therapeutic developers to decide which gene to focus and who to contact for patient access. Dr. Iwata has worked with Dr. Gyan Prakash since 2013 to build eye genetic research programs in Asia and other parts of the world. This book is not only a landmark achievement for GEGC, but it serves as a guiding document for all future collaborative research programs for the Global Eye Genetics Consortium.

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