Essentials in Ophthalmology
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Gyan Prakash
Takeshi Iwata *Editors*

Advances in Vision Research, Volume III

Genetic Eye Research around the Globe

Springer

Essentials in Ophthalmology

Series Editor

Arun D. Singh
Cleveland Clinic Foundation
Cole Eye Institute
Cleveland, OH
USA

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Gyan Prakash • Takeshi Iwata
Editors

Advances in Vision Research, Volume III

Genetic Eye Research around the Globe

Springer

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

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

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Volume 3 is being dedicated to our family members and the research scientists working in the field of vision research

Foreword

Genetics and medical genomics are fast becoming important and essential tools in ophthalmic practice with the past several decades being highly productive for vision community stakeholders. Individuals affected by inherited eye disease, their physicians, and vision researchers are working together to find treatments and cures for these diseases. The completion of the human genome in 2003 accelerated the pace of identifying genetic mutations that cause monogenic disease. It also sped the development of new tools and techniques to define the genetic basis of common complex diseases, such as age-related macular degeneration (AMD) and glaucoma.

In monogenic diseases, a mutation in a single gene is sufficient to define the risk of developing the disease. Gene identification sheds light on the cellular mechanisms of the disease and facilitates the search for effective intervention, but the relationship between genes and disease presentation is not always straightforward. While Stargardt macular dystrophy results from mutations in the *ABCA4* gene and lattice corneal dystrophy results from mutations in the *TGFBI* (*BIGH3*) gene, the relationship between a gene and disease is more complex than a simple one-to-one correspondence. Consider for example X-linked retinitis pigmentosa (XLRP), in which mutations in multiple different genes on the X-chromosome lead to the same clinical disease state with only slight differences. For instance, mutations in the *RP2* gene lying at Xp11.3 and mutations in the *RPGR* (*RP3*) gene lying at Xp21.1 are clinically similar in general. Therefore, making a clinical diagnosis of XLRP does not point directly to either gene as the molecular culprit. The distinction is made by genetic analysis. Molecular therapies of the future will likely treat these two forms of XLRP differently by targeting their distinct pathophysiological cellular mechanisms. Other ocular diseases provide similar examples in which multiple, distinct, different genes share similar overlapping clinical phenotypes. Conversely, different mutations within the same gene can lead to clinically different diseases. *PRPH2* is associated with various retinopathies including macular dystrophy, cone-rod dystrophy, and retinitis pigmentosa. To better understand the entire phenotypic range and molecular etiology of inherited eye disease, there needs to be a concerted effort around the world to work together and compile information especially from the rare disease populations. We need to know the effects of the environment as well as modifier genes. For this, we need data. A lot of data. Not just genetic data, but detailed clinical data as well.

One way to potentially achieve these goals is through the activities of the Global Eye Genetics Consortium (GEGC), which arose out of the Asian Eye Genetics Consortium (AEGC), established in 2014. The signing of an international research collaboration between the National Eye Institute (NEI), National Institutes of Health, and National Institute of Sensory Organs (NISO), National Hospital Organization Tokyo Medical Center, led to the formation of the AEGC to support international scientific cooperation, collaborative research, training, and data sharing. AEGC focused on genetic eye research in Asia, the most populated region of the world where data on genetic variation in inherited eye diseases are limited. The current peer-reviewed literature mostly contains genetic information from patients of European descent. Over time, AEGC brought together over 150 eye researchers from 20 countries interested in collaborative international eye research on individuals affected by inherited eye disease in Asia. This is a perfect example of "if you build it, they will come." Because of the many requests received by the AEGC for entry into the consortium, on May 2, 2018, its members unanimously voted to remove the regional restriction of Asia and expand research activities to the entire globe. Hence, the GEGC was born and now boasts >200 members from >30 countries. The GEGC has the potential to accelerate collaborative international genetic eye research and generate useful new scientific data. The only way for this to be achieved is for its members to remain committed to working together and agreeing on common procedures for data capture, collection, and sharing. GEGC's current activities are focused toward the development of a worldwide database for GEGC members. The GEGC has an opportunity to establish partnerships among scientists, governments, companies, and non-government organizations to support research programs for human health and understanding of the biology of eye diseases.

Far-reaching goals require commitment and adherence to shared practices. For GEGC members to reach their goals, agreed-upon 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 might require. In some instances, it may be enough to know whether a patient had an abnormal electroretinogram (ERG), whereas in others, it will be important to know the specific ERG wave values and times. The use of CDEs, shared data dictionaries, and ontologies will allow users to capture and compare clinical research data methodically, lowering the barrier for comparative data analysis. The 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 the globe. Agreed-upon usage of data collection techniques will allow for better machine learning opportunities. Technology paves the way for new advances, but we must be smart enough to capitalize on them.

There is substantial clinical work ahead of us to define the gene–disease–mechanism relationships. Understanding the genetic relationships will provide important clues into the cell biology and pathophysiology of disease genes and yield improved therapeutics for affected individuals. To be ultimately useful, we will need to construct a systematic medical compendium of

genotype–phenotype descriptions. The potential power of the GEGC is that it can leverage local investments, work on problems of local domestic concern through international collaboration, have access to meta-data, and increase patient participation in gene-based trials, which is especially important for rare disease populations. With over 200 authors across three published books, the GEGC is well on its way toward making its mark in genomic medicine.

Santa Tumminia
National Eye Institute, National Institutes of Health
Bethesda, MD, USA
January 13, 2020

Preface: Recognizing the Special Year 2020

The Global Eye Genetics Consortium (GEGC) represents the global collaboration for finding solution to the eye diseases encompassing many collaborative activities of genetic eye research, training and mentoring opportunities for the next generation of scientists, comprehensive characterization and cataloging of the eye diseases, developing a phenotype–genotype database for diagnosing eye diseases, and defining new and effective standard operating procedures for developing new diagnostics tools and therapeutics for eye diseases and tools for prevention of blindness around the world. This mandate has previously been reflected in *Advances in Vision Research*, Volumes 1 and 2, published in 2017 and 2018. The publication of Volume 3 in the year 2020 marks a very special event in the history for all of us working in eye research. It is not only that the publication comes out in the year 2020, a standard mark for vision heath, but also for the fact that 5 years of accelerated growth since the establishment of Asian Eye Genetics Consortium (AEGC), now GEGC, has given the international researchers an avenue to work together and share the progress on a common global platform. Recognizing the highly globalized nature of research and innovation in the modern times, eye researchers are seeking to collaborate more closely with their counterparts in many parts of the world. As described in several chapters of this volume, there are numerous shared challenges and potential areas of cooperation and mutual learning in various subdisciplines of eye research and potential opportunities to prevent avoidable blindness. These issues have better prospects to be resolved with international collaborations driven by the quality of science being conducted by individual investigators and teams of investigators.

Advances in Vision Research—Volume 3 makes an attempt to describe many facets of genetic eye research. We have the great honor and privilege to highlight this work on many research programs of the investigators from across the globe. We have assembled more than 100 leading researchers from the fields of biochemistry, molecular biology, human genetics, ophthalmology, sensory sciences, clinical research, and other disciplines of biomedical sciences to present the current status of the growing field of genetic eye research. Our hope is that the volume provides a strong background for all researchers, clinicians, clinical researchers, and allied eye health professionals with an interest in eye diseases and prevention of blindness. We also hope that the ideas presented here will accelerate the pursuit of high-quality research to further develop our understanding of eye diseases.

The chapter authors fully assume all responsibilities for the contents, materials, results, interpretations, opinions, discussions, and write-up of their scientific research and findings. As the volume editors, we have neither interfered with any presentations nor verified any materials covered in the book chapters and, therefore, do not assume any responsibilities, direct, indirect, or implied, for the chapter contents in any way of fashion. We were truly privileged to have worked with a group of authors who are recognized leaders in their respective fields and who willingly gave their valuable time to contribute to this volume despite their busy schedules. We are forever in their debt.

This volume and the series of books in *Advances in Vision Research* would not have become a reality without the support, encouragement, and assistance of several family members, GEGC executives, several peers, and distinguished colleagues. Dr. Arun Singh of the Cleveland Clinic, the series editor, provided the support for inclusion in his acclaimed series. Mr. Toshiro Mikami, Mr. Chicko Watanabe, and Mr. Rakesh Kumar Jotheeswaran at Springer Nature provided the impetus and excellent program support for this volume. The GEGC Executives, Prof. S. Natarajan, Prof. Paul N. Baird, and Prof. Calvin Pang, served as the section editors and worked with us diligently in preparing the book. We are very grateful to Dr. Santa Tumminia, Director (Acting) of National Eye Institute—National Institutes of Health in the USA, for her constant encouragement as a cofounder of GEGC with us and her support throughout the last 5 years of the global outreach and for all three volumes of the series. We wish to acknowledge the continued support of the management of National Eye Institute—National Institutes of Health in the USA and National Hospital Organization Tokyo Medical Center in Japan. Finally, and most importantly, we are truly indebted to our family members, Dr. Savita Prakash, Dr. Fumino Iwata, Dr. Shivaani Prakash, Gary Prakash, and Dr. Stefani Su, 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 manuscript together.

We have come a long way since the humble beginning of AEGC/GEGC in 2014 in a small cafeteria in the outskirts of Colombo, Sri Lanka, where the idea of AEGC/GEGC was born. GEGC in the year 2020 represents a consortium of over 200 eye researchers from more than 30 countries dedicated to conducting high-quality research and international collaboration for the genetic eye research and prevention of blindness. We sincerely hope that additional motivated researchers across the globe come together to work collaboratively and produce the highest quality of scientific research for improvement of eye health.

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

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Vice-President, Global Eye Genetics Consortium
NHMRC Senior Research Fellow
Department of Surgery, Ophthalmology
Faculty of Medicine, Dentistry and Health Sciences
The University of Melbourne
Royal Victorian Eye and Ear Hospital, East Melbourne, Victoria
Australia

Prof. S. Natarajan, MD
Secretary General, Global Eye Genetics Consortium
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President, O M A G, Organized Medicine Guild
Immediate Past President, All India Ophthalmological Society
Chairman and Managing Director - Aditya Jyot Eye Hospital Pvt. Ltd.
Trustee, International Council of Ophthalmology
President, APOTS, SN ALUMNI
Immediate Past President, OTSI,
Board Member, RWC, ISOT
Managing Trustee, Aditya Jyot Foundation for Twinkling Little Eyes (Not For Profit NGO)
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Chairman, Scientific Advisory Board, Global Eye Genetics Consortium
S.H. Ho Research Professor of Visual Sciences, The Chinese University of Hong Kong
Director, Joint Shantou International Eye Centre of Shantou University and The Chinese University of Hong Kong
Hong Kong Eye Hospital, Kowloon, Hong Kong

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1 Expansion of Asian Eye Genetics Consortium (AEGC) to Global Eye Genetics Consortium (GEGC), Introduction of a Global Phenotype-Genotype Database "GenEye" and Launch of New Training Programs at National Eye Institute (NEI)

Takeshi Iwata, Gyan Prakash, Paul N. Baird, S. Natarajan, and Calvin Pang

Abstract

The Asian Eye Genetics Consortium (AGEC) established in 2014 brought collective thinking and ideas of the Asian and non-Asian researchers who have an interest in genetic eye research. As the consortium grew, requests to join the consortium from outside of Asia were increasing. During the AGEC meeting at ARVO 2018 in Honolulu, USA, the members unanimously voted to expand the consortium activities beyond Asia and explore unique phenotype and genotype populations in the rest of the world, particularly in Africa and South America. The consortium was renamed the Global Eye Genetics Consortium (GEGC, https://gegc.org) by the general membership. The consortium aims were adjusted and the new GEGC phenotype-genotype database GenEye (https://geneye.kankakuki.jp) was constructed to collect and catalog genetic eye diseases at global scale. GEGC membership has grown to over 200 from five continents, performing GEGC meetings and sessions during ARVO, AIOS, APAO, WOC, and ISER meetings. A number of scientific collaboration and young investigator visiting programs have been successfully launched over the past 6 years.

Keywords

Eye · Genetics · Database · Asia · Africa South America · Global

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

G. Prakash
National Eye Institute, National Institutes of Health, Bethesda, MD, USA

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

S. Natarajan
Aditya Jyot Eye Research Institute and Foundation, Mumbai, India

C. Pang
Chinese University of Hong Kong, Hong Kong, China

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1.1 Expansion of the Consortium from Asia to Global: Launch of the Global Eye Genetics Consortium (GEGC)

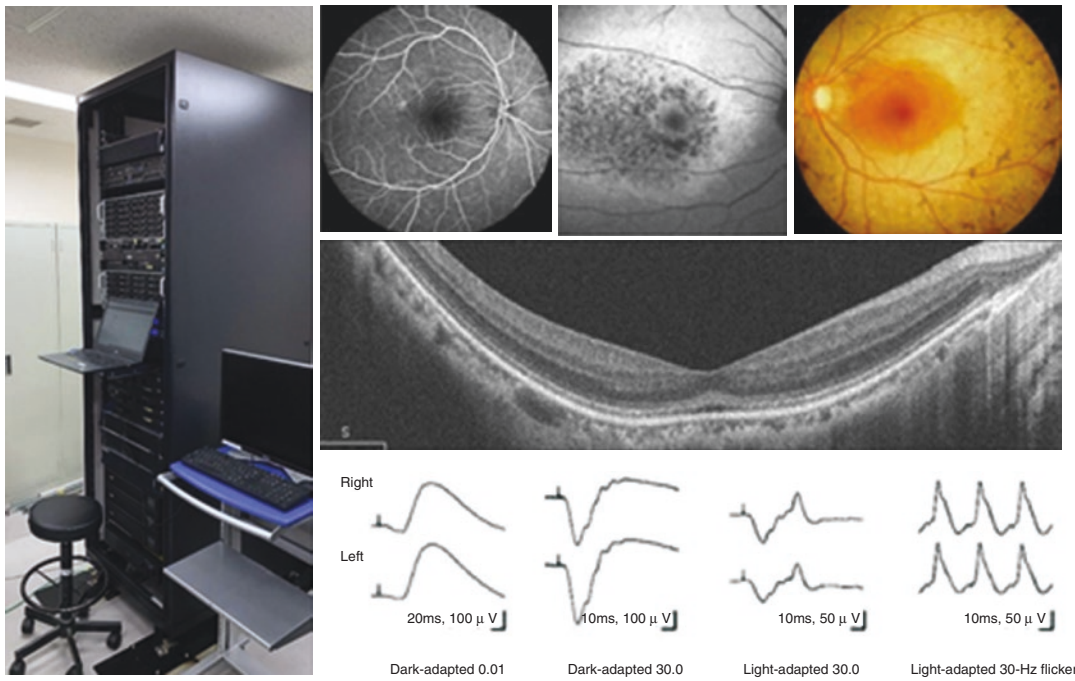
The annual meeting of AEGC was held on May 2, 2018, during the ARVO meeting at Hawaii Convention Center, Honolulu. The status of AEGC database construction was given by Dr. Takeshi Iwata from the National Institute of Sensory Organs (NISO), Japan, followed by an introduction of the European Retinal Disease Consortium by Dr. Frans Cremers and Dr. Susanne Roosing from the Department of Human Genetics, Radbound University Medical Center, Netherlands. Dr. Gyan Prakash from the National Eye Institute (NEI), USA reported the successful publication of Springer Nature, Advances in Vision Research, Volume II, and inviting ideas for Volume III.

Dr. Iwata and Dr. Prakash explained the increase of interest to join AEGC from researchers in Africa, South America, and other parts of the world, and proposed the consortium operation to go global. By a unanimous vote, the global operation was approved, and the consortium name was changed to the GEGC under the same governance structure.

Dr. S. Natarajan from Aditya Jyot Eye Hospital from India gave a status report on the establishment of a new eye genetics research unit in Mumbai followed by another status report from Dr. Kaoru Fujinami from NISO about the East Asia Inherited Retinal Diseases Studies. Mrs. Pamela Sieving from NEI gave a talk on the activity of genetic eye studies in Asia using the bibliometric analysis and showed how this analysis can be used at global scale. The final two talks were about clinical trials in the UK and China. Dr. Rupert Strauss, MD, Moorefields Eye Hospital, University College of London, UK gave a talk on the "ProgStar: International study of Stargardt Disease," explaining the challenges to standardize and unite the group. Dr. Zheng Qin Yin from Southwest Eye Hospital/Southwest Hospital, Third Military Medical University, China discussed development in gene therapy of the eye diseases in China.

1.2 Launch of GEGC Phenotype-Genotype Database "GenEye"

From the early stage of AEGC, the phenotype-genotype database was considered essential for its operation. In 2019, a new phenotype-genotype database, GenEye (https://geneye.kankakuki.jp) was developed at NISO. The database currently contains three diseases including inherited retinal diseases (IRD), age-related macular degeneration (AMD), and glaucoma. The international standard Human Phenotype Ontology (HPO) term is used throughout the database to describe the patient phenotype. Authentication is required for the user to restrict data viewing only to the collaborators before publication.



To facilitate the best design of the GEGC 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. These included meetings with Dr. Takeshi Iwata from NISO, providing an example of the Japanese IRD database, meeting with Dr. Andrew Webster from Moorefields Eye Hospital about the UK IRD database, and meetings with Dr. Kerry Goetz and Dr. Santa Tumminia from NEI about the US eyeGENE database.

GEGC is currently promoting research groups with no access to the phenotype-genotype database to collect and store patient data into GenEye. The first phase of GenEye is to accumulate phenotype data from Asia, Africa, and South America into GenEye and later plan for whole genome analysis by international collaboration and funding. Asia, Africa, and South America are under-represented in the global knowledge base, however these regions are some of the most populated and information gained from these regions will greatly advance our understanding of molecular mechanisms and pathobiology of genetic eye diseases. The outcomes of the GenEye are expected to catalog genome mutations and variants associated with specific genetic eye disease across Asia, Africa, and South America. It will also provide big data sets at global scale for artificial intelligence (AI) and machine learning to develop future diagnostic systems without the presence of an ophthalmologist. GEGC is also expected to work with drug companies planning for clinical trials at a global scale. GenEye will quickly identify patients with the same disease and genome mutation for each location, benefiting the companies via a shorter time period to decide where to set up the center for maximum patient recruitment.

1.3 The Updated Aim, Management, and Budget for GEGC

1. Share genetic information to isolate common genetic variants associated with eye diseases
2. Establish cost-effective genetic analysis and accurate diagnosis for grouping of genetic eye diseases
3. Develop research-oriented database to collect and catalog genetic eye diseases at global scale
4. Support and foster global collaboration and scientific exchanges for the advancement of genetic eye research
5. Collaborate with other international and regional organizations with similar goals
6. Organize regional congresses and other educational and scientific activities to promote goals of the consortium

The aim of GEGC was re-adjusted to fulfill the need for the global operation. Recruitment of members to the establishment of Scientific Committee, Clinical Diagnostic Committee, Ethics Committee, and additional Vice Presidents from Africa and South America, are being discussed. At the GEGC executive meeting at APAO in Bangkok, Thailand on March 7, 2019, Dr. Calvin Pang from the Chinese University of Hong Kong, China was appointed as Chief of GEGC Scientific Committee. These committees will play an important role to keep GEGC operation at global standard.

Self-sustainable budget is an important aspect of collaboration in GEGC. Most research grants in the countries limit DNA sequencing to local patients and do not allow any import or export of genetic materials for patients from abroad. An international collaboration grant is jointly planned for submission to the Japan Society for the Promotion of Science (JSPS) and Department of Science and Technology (DST) of India. Additional scientific grant submissions are planned for NIH (USA).

A multifaceted global effort like GEGC has the potential to accelerate the collaborative genetic eye research in generating useful new scientific data to help our understanding of genetics in 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 has created a wide opportunity to establish partnerships among scientists, governments, companies, and non-government organizations to support research programs for understanding the biology of eye diseases on a global level.

1.4 GEGC Lab Exchange Program for Young Scientists

During the last 7 years, eight researchers have used the Lab Exchange Program of AEGC/GEGC to visit foreign labs to experience how the genetic research is operated. This program provides the opportunity for young scientists and strengthen future collaborations on genetic eye research around the globe. The hands-on training for the GEGC members are one of the key goals of this Lab Exchange Program. Such programs have helped establishment of the new GEGC eye genetic laboratories in Mumbai, India. GEGC is working on government funding or corporate support to conduct whole genome sequence on DNA samples from the countries that have limited or no research funding locally. Over 200 eye researchers from more than 30 countries have become GEGC members since its inception. The members are currently interacting and collaborating to develop research programs to catalog and share disease-causing gene mutations of genetic eye diseases in Asia, Pacific, Africa, and South America.

1.5 GEGC as a Member of the International Council of Ophthalmology (ICO)

The first GEGC annual meeting was held on April 29, 2019, during the ARVO meeting in Vancouver, Canada. Representative from 18 countries gathered to this meeting. After the introduction of GEGC, President of International Council of Ophthalmology (ICO), Dr. Peter Wiedemann and Vice-President, Dr. Neeru Gupta welcomed GEGC as a new member of the ICO. GEGC will now work with ICO to expand in Africa and South America. Mrs. Sieving gave a talk about the scientific activities in Africa and South America based on the bibliometric analysis in these regions.

Dr. Margaret DeAngelis, a member of the International AMD Consortium, University of Utah, USA gave a talk on how to organize international consortium, showing pitfalls that she experienced and how the consortium dealt with the difficulty. The ongoing construction of the new GEGC phenotype-genotype database "GenEye" was introduced by Dr. Iwata. To meet the next level of research at a global scale, establishment of Scientific Committee, Clinical Diagnostic Committee and Ethics Committee were discussed with the team consisting of Dr. Iwata, Dr. Prakash, Dr. Baird, Dr. Natarajan, Dr. Pang, Dr. Tumminia, and Dr. Goetz.

1.6 New International Training Programs/Fellowships in Genetic Eye Research at National Institutes of Health: National Eye Institute in the USA

1.6.1 New Program Launch: NEI-ICO Fellowship for International Fellows from Lower- and Middle-Income Countries (LMIC)

NEI has just started a new ICO Fellowship being managed by the International Council of Ophthalmology (ICO). The new program was kicked off at ARVO-Vancouver, Canada in May 2019. The fellowship is directed for early-career meritorious *candidates/clinicians from* LMICs to have one fellowship per year generally for one-year duration at NEI in Bethesda, Maryland, the USA beginning in 2020.

1.6.2 Expansion of International Genomics Fellowship at National Eye Institute in the USA

NEI is working in collaboration with National Human Genome Research Institute (NHGRI) at NIH to organize the month-long fellowship for early-career lab/research and clinical scientists from the lower- and middle-income countries. The fellowship started 5 years back and is likely to continue based on the funding. In 2019, NEI became the largest sponsor of international fellows (total 5 in 2019) at the NIH wide International Genomics Summit. Over the past 5 years and from the beginning of this program, NEI-NIH has sponsored and trained ten international fellows since the launch of GEGC. The early-career scientists have come from several other countries, including India, Ukraine, Turkey, Pakistan, Bangladesh, Nigeria, Argentina, and Mexico.

1.7 Updates on Other GEGC Sessions and Meetings During 2018 to 2019

1.7.1 GEGC Session at SAARC Academy of Ophthalmology 2018

The GEGC session was held during the South Asian Association for Regional Cooperation (SAARC) Academy of Ophthalmology (SAO) meeting on June 22, 2018, at Hotel Yak and Yeti, Kathmandu, Nepal. Over 80 people attended the meeting.

After the SAO meeting, Dr. Takeshi Iwata and Dr. Paul Baird visited the B. P. Eye Foundation and the Tilganga Institute of Ophthalmology in Kathmandu.

1.7.2 GEGC Meeting at World Ophthalmology Congress 2018

The GEGC meeting was held during the World Ophthalmology Congress on June 18, 2018, at Barcelona Convention Center in Barcelona, Spain. Introductory comments were made by Dr. Hugh Taylor and Dr. Peter Wiedemann, the President and incoming President of ICO, respectively. Dr. Gyan Prakash from NEI, USA, and Dr. Paul Baird from the University of Melbourne, Australia gave the introduction of GEGC and the update on the Springer Nature Advances in Vision Research Volume II. Dr. Calvin Pang from the Chinese University of Hong Kong gave an overview of the research in retinal diseases of China followed by Dr. Paisan Ruamviboonsuk, applying AI for screening diabetic retinopathy in Thailand. The last talk was given by Mrs. Pamela Sieving about the bibliometric analysis of the AEGC research. Dr. S. Natarajan from Aditya Jyot Foundation for Twinkling Little Eyes, India did the closing remarks.

1.7.3 Foundation of the GEGC China Branch

On June 28, 2018, at Kempinski Hotel Chongqing, China, a meeting for the foundation of the GEGC China Branch was held by the leadership of Dr. Zheng Qin Yin from Southwest Eye Hospital/ Southwest Hospital, Third Military Medical University, China and Dr. Qingjiong Zhang from Zhongshan Ophthalmic Center, Sun Yat-sen University, China.

1.7.4 GEGC Session at Asia Pacific Academy of Ophthalmology 2019

The GEGC Symposium was held on March 6, 2019, during the Asia Pacific Academy of Ophthalmology at Bangkok Convention Center in Thailand. The following talks were given by the speakers. Novel Genes Identified for Inherited Retinal Diseases in Asian Population by Takeshi Iwata, From Genome-wide Association Studies to Mendelian Randomization: Opportunities for Understanding Ocular Disease Causality by Dr. Ching-Yu Cheng, Can an Ophthalmologist diagnose a Rare Genetic Syndrome of Werner by Dr. Ahmed Reda, Leber's Congenital Amaurosis in China by Dr. Zheng Qin Yin, Role of GEGC, India Chapter in Promoting Eye Research by Dr. S. Natarajan, Successful Treatment of Secondary Choroidal Neovascularization Associated with Best Vitelliform Dystrophy with Anti-VEGF Therapy by Dr. Tharikarn Sujirakul, presentation by Dr. Amir Hossein Mahmoudi, and Research as a Tool for Serving Community Eye Health Needs and Building International Collaboration by Dr. Gyan Prakash.

GEGC continues to make special efforts in organizing and coordinating regional, national, and international conferences and symposia in order to bring research collaborators to expand the research and training activities. In the past 5 years, NEI in the USA has already trained a good number of next generations eye geneticists from lower- and middle-income countries. The trained scientists have returned to their home countries and organizations and have started setting up new labs and programs further enhancing various activities of GEGC. Additional new programs, symposia, and conferences have been planned in the US, India, Japan, South Africa, Argentina, China, and several other countries providing world-class opportunities at the regional level for the GEGC plans to grow and achieve its goals.

2 Global Women's Eye Health: A Genetic Epidemiologic Perspective

Patrice M. Hicks, Leah A. Owen, and Margaret M. DeAngelis

Abstract

Globally, women experience a greater burden of eye disease as compared to men. Women around the world face different obstacles in maintaining both healthy vision and prevention of vision loss. Understanding women's eye health can be a challenge because risk can vary based on demographic, environmental, genetic, and social factors. Women also experience varying risk for visual impairment in different stages of their lives such as pregnancy, menopause, and post-menopause. Moreover, women experience a greater risk of visual impairment because of where they live in terms of resources and social and cultural norms. Furthermore, genetics can increase women's risk for visual impairment due to heritable ocular conditions and gene-environment interactions. Overall, due to these factors, women have differences in health needs that are less understood and can cause health inequity for women across the globe. Understanding these unique health needs can only be achieved by research driven to address these issues and why it is essential for women to take part in research and clinical studies. Thus addressing these unique health needs are essential to provide opportunity for women around the world to have both adequate and relevant health care, targeted preventative services, and management services to avoid lifelong visual impairment.

Keywords

Women's Health · Eye disease · Visual impairment · Epidemiology · Genetics · Eye care · Underserved populations · Global health

P. M. Hicks
Department of Ophthalmology and Visual Sciences, University of Utah, Salt Lake City, UT, USA

Department of Population Health Sciences, University of Utah, Salt Lake City, UT, USA
e-mail: Patrice.Hicks@hsc.utah.edu

L. A. Owen
Department of Ophthalmology and Visual Sciences, University of Utah, Salt Lake City, UT, USA
e-mail: Leah.Owen@hsc.utah.edu

M. M. DeAngelis (✉)
Department of Ophthalmology and Visual Sciences, University of Utah, Salt Lake City, UT, USA

Department of Population Health Sciences, University of Utah, Salt Lake City, UT, USA

Department of Ophthalmology, University of Buffalo Jacobs School of Medicine & Biomedical Sciences, Buffalo, NY, USA
e-mail: margaret.deangelis@utah.edu

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2.1 Current Review of Women's Eye Health

It has been well established that women in general, have contrasting health needs compared to their male counterparts [1–4]. To date, these differences in health needs have been understudied and because of this woman face a more significant amount of burden due to health inequity across the globe [5–7]. These gender differences as they pertain to specific health needs may arise due to differences in socially constructed roles and socially constructed relationships in terms of a person's values, relative power, and behavior [2, 8]. Therefore, women experience differences in health risks and access to health care. The eye can be an indicator of systemic disease, such as cardiovascular risk factors including, hypertensive and diabetic retinopathy [9–12] These blinding eye diseases can manifest within the retina before it is diagnosed systemically [13, 14]. It has been demonstrated that for many cases, diabetic and hypertensive retinopathy goes undiagnosed until an individual receives a retinal examination [9–11]. In the last 20 years, research continues to show that women are more greatly affected by both vision loss and blindness compared to their male counterparts [15]. When observing the public health issue of global blindness, women account for 55% to 66.7% percent of those that are blind as compared to men [15–18]. Recently more focused research has been conducted within the area of gender and sex differences within health outcomes that includes the biological sex differences as wells as the social factors [2, 19, 20]. Overarching social factors that can affect women's health are outlined in Fig. 2.2 and overarching biological factors are outlines in Fig. 2.3. To illustrate an example seen in Fig. 2.1, consider Shuri, a 24-year-old woman who lives within West Africa, where visual impairment, blindness, and eye disease is a significant public health problem [21–23]. Now consider the biological and social factors that differ between her and Ayo, a 24-year-old male living in the same region of Africa as Shuri. The most apparent difference between Ayo and Shuri is that Shuri is a woman. Not only would Shuri, as a women face unique health challenges about her overall eye health and vision because of biological differences between her and Ayo, but she may also experience different social factors as well. Next, let us consider Shuri and Ayo's differences from a biological stance. Shuri may experience these biological differences because of hormonal differences for example, during pregnancy, menopause, and/or during the years after she experiences menopause, post-menopause. Finally, let us consider the differences in maintaining a healthy vision between Ayo and Shuri in terms of social differences. Shuri's responsibility as a caretaker to her home may make it more challenging to leave her home and travel to where eye care services are available. Ayo, as a male, may not have these same familial responsibilities and may be able to travel to access his eye care services more efficiently. These differences create unequal abilities to maintain healthy eyes and vision for Shuri. These differences may cause health disparities for women within eye health and eye care.

There are excellent reviews that have been published with respect to women's eye health

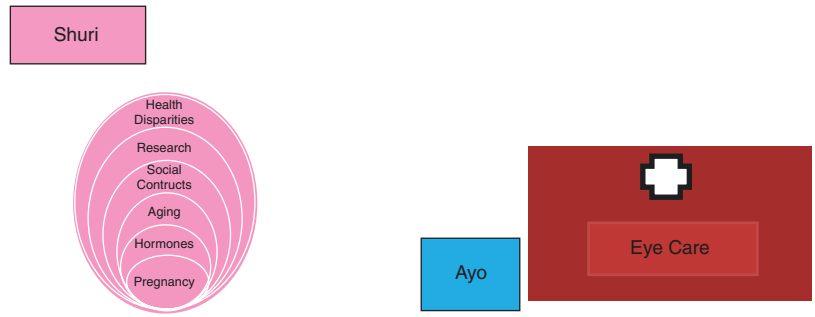

Fig. 2.1 Women's more significant barriers to healthy eye and vision

[16, 24, 25]. These reviews include Clayton and Davis 2015 that address the diseases that affect women more and ultimately their eye health, as well as challenges and further needs for women's eye health [24]. Doyal and Das-Bhaumik describe gender differences in blindness and visual impairment between men and women. They also, discuss the inequalities and obstacles that women face in receiving eye and vision care [16]. Our review will expand on prior knowledge regarding eye disease to include vision health challenges for women as compared to men by continent. Moreover, we will discuss challenges to eye care services that require further research efforts to address these inequalities for women around the globe. This review will build upon the challenges women face around the world for maintaining both healthy vision and prevention of vision loss. Moreover, it will explicitly discuss eye diseases that affect women disproportionally including dry eye disease [26–28], primary angle closure glaucoma (PACG) [29–32], cataracts [33–35], age-related macular degeneration (AMD) [36–38] and myopia [39, 40], the barriers that women face accessing vision care including cost [41], social norms [42, 43], living in a low-resource country [42, 44] and cultural norms [42, 43], research barriers to women's vision health including historically less representation in research [45, 46], enrollment in research studies [45, 47, 48] and retention in research studies [45, 47, 48], the effects of differing hormones on vision within women that occur during pregnancy [49], menopause [50, 51] and post-menopause [50, 52], and unique genetic factors associated with female gender [53, 54]. Additional risk factors that will be considered include, women's vision health as they age compared to men because they tend to live longer [16, 18, 55]. Recent initiatives to implement the needs of women in research include the Women's Eye Health and Safety month [56, 57], Women's Eye Health lead by Harvard University [58, 59], and the Fred Hallows Foundation's approach to addressing cataracts [60, 61], and the Office of Research on Women's Health. [62].

2.2 Overall Global Blindness

Blindness and visual impairment is a public health burden for women around the world as the female sex is more highly prevalent compared to male sex (55% to 66.7% total blindness and visual impairment) as presented in Table 2.1 [33, 94–96]. Women experience a higher burden of certain eye diseases as compared to men [29, 33, 34, 97, 98]. These diseases include the increased burden of cataracts [33–35], angle closure glaucoma [29–32], and dry eye disease [26–28].Other conditions may be more prevalent in women, in specific populations. There is Epidemiological evidence that eye disease has sex-associated risk. This sex-associated risk has been found in women's estrogen pathway and associated with eye disease risk such as cataracts [99, 100]. A review by Zetburg observed that estrogen was protective against cataracts [99]. This conclusion was based on findings of the Blue Mountain Eye Study, the Beaver Dam eye study, and the Salisbury Eye Study, which included both women and men [99]. These studies had participants that were aged 43 years and older. They were conducted in Australia, Wisconsin and Maryland, and ascertained participants of primarily of Caucasian [99]. Some studies have found a higher risk for eye disease within women based on clinical subtype, such as in the case of age-related macular degeneration [63, 64, 66]. For example, Rudnicka et al. found that women had a potentially higher risk of neovascular AMD, but not overall AMD prevalence [66]. These findings were based on a meta-analysis that included 25 population-based studies within 31 populations. There were 51,173 participants all over the age of 40 years, predomi-

Table 2.1 Reports of higher prevalence of blindness/visual impairment in women around the globe

Population	Higher prevalence in women
Africa [7, 125, 136]	Yes [7, 63, 64]
Asia [7, 125]	Yes [7, 64, 65]
Australia [125, 137, 190]	Yes [64–66]
Europe [190, 205, 206]	Yes [23, 65, 67]
North America [218, 219, 220]	Yes [65, 68, 69]
South America [137, 190]	Yes [65, 66, 70]
Industrialized countries [125]	Yes [7, 63, 64]

nantly of European ancestry, but also included Blacks, Hispanics, Eastern Asians, and Mexican Americans. Within the 25 studies, 19 of them included both men and women while one study only included men and 5 studies did not include the gender of their study participants. Future studies may consider focus on making sure that women are equally represented within their research studies. Studies have also shown that Glaucoma may be influenced by estrogen pathways [72, 101–105]. Newman-Casey et al. conducted a retrospective longitudinal cohort analysis, which included 152,163 women aged 50 years and older [106]. The study found that women who used postmenopausal hormones were less likely to develop primary open-angle glaucoma [106]. Hulsman et al. conducted a study using the data from the Rotterdam study, which is a population-based prospective study [107]. The specific study included 3078 women aged 55 years and older [107]. The study found that women had decreased odds for open-angle glaucoma if they were exposed to endogenous hormones, specifically their odds decreased by 5% per year of exposure [107]. If the women were at the age of menopause then it decreased by 6% per year of exposure [107]. Thus research focused on glaucoma risk for women may want to include quantitative levels of endogenous and exogenous hormone levels. In addition, higher levels of exogenous and endogenous hormones are associated with a greater risk of development of breast cancer [108]. Thus, a monitoring system of glaucoma may help to inform risk of additional diseases. Uveitis can cause inflammation of the tissue of the eye [109]. Therefore, Uveitis can cause decreased vision as well as vision loss [109, 110]. Risk factors for the disease include injury to the eye, autoimmune disorders, tumors, or infections within the eye or the body [109, 110]. Women are more likely to develop non-infectious uveitis as compared to men [111]. It is hypothesized that women may be more likely to develop autoimmune diseases in general, which as previously stated are a risk factor for Uveitis [111]. Yang et al. found that of 45 men and 53 females examined of Chinese ancestry, that only women with anterior uveitis had an increased allele frequency of CFH-rs800292G allele as compared to women without anterior uveitis [112]. Analysis of the Tsuruoka Cohort Study that included adults of both sexes (1815 men and 2173 women) aged 35 to 74 years in Japan, found that having rs800292 (I62V) polymorphism may increase the risk for developing the early form of age-related macular degeneration for women as compared to men [113]. This same study also found that having the rs10490924 in the ARMS2 gene may also increase the risk for developing early age-related macular degeneration in women as compared to men [113]. Women also have a higher burden of infectious eye disease in low-resource countries across the world, such in the case of Trachoma were it can be seen two to six times more in women [114–117]. Trachoma, which is an infection that occurs in the eye when the bacteria *C. trachomatis* is transferred by hand, cloth, or flies to the eye. Trachoma occurs mostly due to household crowding and poor access and usage of water in these highly affected areas [115, 118–120]. This is the case in rural areas of Africa, Asia, Central America, South America, Australia, and the Middle East [114–117]. The prevalence of Trachoma is not isolated to one specific continent but does become more pronounced if a woman is a member of a marginalized group [117, 121–123]. Women can be marginalized because simply because they live in poverty regardless of geographic location [115, 117, 123, 124]. Ninety percent of women who are blind live in poverty [125]. Their household income may instead be used for the needs of boys or men within their household, rather than the girls or women. In some cases, women's relatives or husband may choose whether or not to spend money on the women's health care needs [16, 126, 127]. In addition, religion can have an impact on the eye care of women around the world. In some instances, it is religiously custom to have extended family make the decisions for both medical treatment and care [128, 129]. These limitations include monetary constraints which cause them to have overcrowding within their homes, limited water supply, male support for health needs, and travel distance to health care and preventative care services. Spending

more time with children can make one more susceptible to having Trachoma then those who do not. In general, women are the primary caretakers of the household and therefore spend more time with children [117, 121–123]. Women also have a higher burden of trichiasis, a site threating outcome from repeated trachoma infection [115, 118–120].Trichiasis occurs when one's eyelashes turn inward and upsets the cornea, by scratching the cornea causing scarring [119]. This may be more prevalent in women because they are the primary caregivers to children and may not be able to make treatment of trichiasis a priority [115, 119]. Trichiasis is found more often in women in countries where Trachoma is a public health problem such as rural and low-resource areas of Africa, Asia, Australia, Central America, South America, and the Middle East [114, 117]. In addition, there is currently no clear biological understanding of why women may be more prone to the development of infection in the eye. It has been hypothesized that women have greater susceptibility to dry eye syndrome, especially in older women, which can cause cornea scarring [130]. Women may be more likely to have corneal damage, which could be why when women have trichiasis they are more likely to have more severe outcomes from the disease as compared to their male counterparts. Visual impairment caused by Myopia and Hyperopia is also seen at a higher frequency in men compared to women [40, 131–133]. Myopia has also been found to progress more rapidly in females as compared to males [71, 134, 135]. This is has been observed in the USA, China, and Columbia [40, 71, 135]. Table 2.1 depicts the prevalence of overall blindness in women by continent while Table 2.2 depicts particular disabling eye conditions for women by continent., that are described in further detail below.

Table 2.2 Reports of greater burden of eye disease and conditions in females around the globe

Population	Eye disease or conditions
Africa [149, 150]	Trachoma/Trichiasis [48, 63, 71]
Africa [139, 146]	Cataract [24, 71, 72]
Asia [173, 188]	Cataract [73–75]
Asia [117, 118, 189]	Trachoma/Trichiasis [48, 52, 76]
Australia [117, 118, 189]	Cataract [77–79]
Australia [198, 199]	Trachoma/Trichiasis [80–82]
Europe [211, 212]	Cataract [83–85]
Europe	Hyperopia [86–88]
North America [236, 242]	Dry Eye Disease [89, 90]
North America [222, 223, 241]	Cataracts [91, 92]
South America [118]	Trachoma/Trichiasis [48, 52, 93]

2.2.1 Africa

Women are more likely to experience blindness in Africa, as compared to men. Women in Africa are still more likely to experience blindness compared to men, even after accounting for age [7, 125, 136]. Results from a meta-analysis conducted by Abou-Gareeb et al. using studies that were population-based and included a clinical examination found a higher prevalence of blindness, determined by a clinical evaluation, for women living in Benin, Congo, Egypt, The Gambia, Kenya, Mali, Morocco, South Africa, Tanzania, and Tunisia [125]. Others have found an overall higher prevalence of blindness in women in Nigeria as compared to men [137, 138]. The same was seen in Ethiopia [137, 139]. Berhane et al. 2007 conducted a cross-sectional study that observed 30,022 participants consisting of both men and women. The study used the national blindness and low-vision survey and observed a higher prevalence of blindness in women [139]. They also observed that most blindness was due to avoidable causes of blindness such as cataract and refractive error [139]. The region in Africa where women experience greater overall visual impartment ratio compared to men is Central Sub-Saharan Africa based on a visual impairment gender ratio [140–143]. Sub-Saharan Africa consists of 46 of Africa's 54 countries and is defined as the countries that are south of the Sahara desert [144]. A contributing factor to greater blindness in women is a lower rate of cataract surgery within the African population [76, 126, 145]. Prevention of blindness due to cataracts by sur-

gery is lower in women compared to men usually due to cultural and social barriers that will be explored later in this review [146–148]. The chronic tropical infectious disease Trachoma is mostly found in Africa compared to other continents. After cataract, trachoma is the second leading cause of blindness overall. In Africa, Women are more affected by trachoma than men and also have a higher prevalence than women from other continents as depicted in Table 2.2. Gender roles have the greatest impact on trachoma in women in Africa, as they are the primary caregivers in the home and have close contact with children [149, 150]. Trachoma is a bacterial infection, so good hygiene practices are essential to limit the risk of contraction of the disease [65, 151, 152]. Villages with scarce resources, such as clean water, can make it difficult for women to be able to use proper hygiene mechanisms to avoid contraction of the disease and since they are spending much of their time in direct contact with children who also do not have access to clean water this just amplifies the problem [153–155]. Men in Africa suffer Onchocerciasis or "river blindness" more than women in Africa because of farming and fishing, but women in Africa face a higher prevalence of river blindness more in Africa compared to other continents around the world [77, 78, 156]. Onchocerciasis is caused by the larva of the worms *Onchocerca Volvulus* that are transmitted to the fly of genius *Simulium* with the most popular being *Simulium damnosum sensus lato*, also known as a blackfly, infecting the eye directly. River blindness is a parasitic disease, and the majority of cases are found within the continent of Africa [77, 157, 158]. The disease is spread through bites from infected blackflies, and there is no current vaccine available to prevent the virus that causes the blinding eye disease [67, 159, 160]. The blackfly breeds in the rivers and the streams of tropical areas of Africa. The World Health Organization has estimated that 99% of people infected with river blindness live in sub-Saharan Africa in the countries of the Democratic Republic of the Congo, Chad, Ethiopia, Cameroon, Angola, Benin, Mali, Niger, Togo, Sudan, Central African Republic, Ghana, Kenya, Uganda, United Republic of Tanzania, Burkina Faso, Republic of Congo, Nigeria, Equatorial Guinea, Liberia, Burundi, Côte d'Ivoire, Guinea-Bissau, Malawi, Mozambique, Rwanda, South Sudan, Sierra Leone, Gabon, Guinea, and Senegal [159, 161]. Agriculture is important for the African economy, and these areas as expected are near the rivers and streams where the blackflies breed. In addition, women and young girls are primarily responsible for the washing of household clothes [159, 161]. Those who wash clothes in the river have an increased risk of contracting the disease because that is where the blackflies breed [162]. There have been efforts in these areas through initiatives such as the African Program for Onchocerciasis Control (APOC) that has greatly reduced the disease [83, 156, 159, 161]. The program closed in 2015, but the World Health Organization still addresses the need of controlling the disease through the Expanded Special Project for the Elimination of Neglected Tropical Diseases (ESPEN) [83, 84]. The treatment for the disease consists of ivermectin which is free of charge to those that need it do address disparities in unequal access [156, 159]. Though there is the treatment that is available, it can be difficult to reach those that need treatment in remote areas and populations due to war [83]. Though men have a higher prevalence, women are disproportionally burdened by the chronic condition of river blindness due to cultural aspects as previously discussed being that they are the primary caregivers to the children in the household and may not prioritize treatment compared to men [77, 78, 156]. The effects of blindness in women can take a greater toll compared to men due to the stigma associated with the disease. Women who go blind from the disease can be looked down upon in terms of human worth because they have gone blind and are unable to complete their routine household obligations such as taking care of the children, housework, and cooking in these rural areas of Sub-Saharan Africa that are most affected by the disease [163–165]. Women with blindness also do not get married or take longer to get married due to the stigma associated with the disease and skin rashes that the disease causes [163–165]. There is also a belief that women with the disease will pass the disease onto their offspring's causing their prospects of marriage to also decrease [163–165]. An education interven-

tion to address these beliefs may be needed to increase education about the disease. If their prospects of marriage decrease their poverty increases especially in a society where women are not seen as equal to men.

It has not been established if women have higher rates of refractive errors compared to men, but women in Africa experience a greater burden of refractive error more than women from other areas around the world that have more greater resources to achieving great vision [166, 167]. This is because in rural areas of Africa they have less access to screenings for refractive error as compared to areas of higher resources such as Canada and the USA [166, 167]. In addition, if they have refractive errors it is more difficult for them to obtain glasses for correction as compared to Women in regions with greater access to eyeglasses and eye health services [166, 167]. Ultimately this decreases their quality of life [167]. Public health efforts in Africa may want to address these eye diseases and barriers to eye care services. These interventions should center on gender-specific needs for women to decrease the prevalence of blindness and visual impairments in regions of Africa that are affected the most [15, 117, 126]. These interventions may best serve the needs of women if they were conducted employing community-based approach that includes culturally appropriate mechanisms that include education of hygiene and transmission of infection [126, 148, 168]. Public Health interventions to address blindness and visual impairment in Women in Africa that are most affected should focus on reduced or no cost, accessibility, education, and social support. Cost should be addressed so that women can have the opportunity to utilize eye care services and surgical treatment for both cataracts and trachoma [126, 168]. In addition, for trachoma programs of prevention and intervention such as surgery for trichiasis, antibiotics, facial cleanliness and environmental improvement (SAFE) which have already been implemented require continuous support to achieve success. This intervention has proven to be successful because it is community based through community involvement and primary health care providers [117, 168]. Roba et al. conducted a cross-sectional study in Ethiopia using survey results to determine the effectiveness of a SAFE intervention [68]. Follow-up surveys consisted of 8358 participants aged 14 years and older and 4535 children aged 1 to 9 years of age that were observed for trachoma [68]. The study found that trachoma trichiasis decreased from 4.6% to 2.9% [68].

The National Institute of Health has partnered with the African Society of Human Genomics and the welcome trust through the Alliance for Accelerating Science in Africa to form the Human Hereditary and Health in Africa (H3Africa) [169]. A collaborative research project "Eyes of Africa: The Genetics of Blindness" will conduct GWAS studies to identify risk SNPs that may be associated with primary open-angle glaucoma (POAG), using POAG cases and controls [69]. The study will be conducted in the African countries of Nigeria, Gambia, South Africa, and Malawi [170]. This study and future studies may illicit clues that provide genetic information on differences between men and women in terms of blinding eye disease in Africa.

There is a need for accessibility interventions that focuses on both access to providers and transportation to services. Creating community efforts to have providers within the community and not only in major cities is critical to making sure everyone has equal access [168]. Global efforts to improve women's eye health include the coordinated approach to a community health program in Uganda, which aims to increase early detection of trachoma screenings through initial consolation [60, 171]. The program occurs in what is considered the dry season for agriculture so that more women can take part in the program because they are not working in the fields as much during this time period [60]. Women may not know that cataracts and trichiasis surgery is available to prevent blindness. Finally, social support at the community level is important to address the burden of blindness and the prevention of visual impairment within populations that are most affected. Future research considers tribes within the community if they exist to best create public health interventions that will meet the needs and wants of each individual tribe within the overall community's health needs [12].

2.2.2 Asia

More women in Asia are blind as compared to men, as demonstrated in Table 2.1. It has been estimated that women in Asia have a 1.41 higher odds of going blind as compared to men in the same continent, even after adjusting for age [7, 125, 171]. A higher burden of blindness for women can be found across the continent of Asia in countries such as China, Lebanon, Nepal, and Saudi Arabia [125]. Though the information is given on sex differences for blindness in India, the findings were indifferent within two different surveys within the meta-analysis conducted by Abou-Gareeb et al. [125] Women living in India are less likely to obtain eye care services due to societal status, especially later in life [171–174]. This may be because women in India are more likely to have less education compared to men in India, decreased ability to financially support themselves, and have lower social status due to the social preference of men in India [175]. It has been noted that women 50 years and older living in Central and South Asia combined have a higher prevalence of blindness as compared to men in the same regions [176, 177]. In addition, overall in East Asia, South-East Asia, South Asia, Central Asia, and high-income Asia Pacific regions of Asia, there is a higher prevalence of blindness in women as compared to men [98, 176, 177]. All of these regions make up the entirety of the continent of Asia.

Visual blindness, which occurs in Asia, has a higher prevalence in women than it does in men of all ages [15, 176]. This is true within the regions of East Asia, South-East Asia, South Asia, Central Asia, and high-income Asia Pacific [137, 176, 178–182]. The region of Asia with the most significant difference between men and women in terms of visual impairment is the high-income Asia Pacific [182]. The region of high-income Asia Pacific includes the countries of Brunei, Japan, and South Korea. With women in Asia being disproportionately burdened by both visual impairment and blindness, a gender focus initiative could be taken by those in public health to improve eye care services within this continent to prevent visual impairment. South Asia has one of the highest prevalence of blindness within its aging population around the world [98]. Women tend to outlive men and so they tend to make up more of the aging population. South Asia has a tremendous social preference towards men, so a public health approach that addresses women's eye care may be implemented within this population to have the greatest impact in address women's eye health within this continent [183].

Primary angle closure glaucoma (PACG) is a form of glaucoma that increases one's ocular pressure and causes half of the blindness from glaucoma worldwide. [184] PACG occurs when the iris is found to block the drainage of the eye through the trabecular meshwork [184, 185]. Women are most likely to develop the disease, especially women of Asian ancestry women [184, 185]. Research studies have found that within the population of Eastern Asian women, there is a higher incidence of primary angle closure glaucoma (PACG) as compared to men [186, 187]. Cheng et al. conducted a meta-analysis that examined 29 population-based studies including 39,180 men and 44,723 women, that included studies that included those 30 years and older, within Asian populations. The analysis found that the highest prevalence of PACG was reported in Japan, followed by China then the Middle East [186]. Glaucoma interventions within these regions may want to focus on PACG as their primary target, as more Asian women are affected by this type of glaucoma compared to Asian men.

Multiple studies have shown that women in India have higher rates of cataracts as compared to men [173, 188]. Pant et al. conducted a study using two population-based surveys conducted in India with 50,344 men and 58,265 women. The research found that females had a higher prevalence of cataracts in both of the surveys and a higher risk for cataracts [173]. Women living in India are also less likely to receive services to treat their cataracts such as cataract surgery. This is due to societal status, their health needs are considered less important as compared to their male counterparts [172, 173]. This may be due to societal structures where women are beneath men, as well as their roles as caregivers within the household and how they are portrayed within

their culture [172]. As we previously discussed the continent of Africa, trachoma trichiasis disproportionally affects the women in Asia because of their close contact with children because they are the primary caregivers within the household [117, 118, 189]. In addition, there are also areas of low resources throughout Asia that increase a women's risk for the disease due to household crowding and poor access and usage of water sources [117, 118]. Women are less likely to seek treatment for this infectious eye disease and have a greater burden from the disease as compared to men because they are less likely to access care for the disease [117]. Due to cultural and societal aspects, which puts their health needs beneath men, women in Asia become blind from the disease for not accessing care [117, 118, 189].

New efforts to address the differences in eye care between Indian men and Indian women may want to be gender-focused to achieve equal access to vision services for women living in India [173]. Community efforts that focus on the cost of services could be addressed, as well as access in terms of availability and transportation especially in rural, low-resource areas within Asia. Similarly to what we have explored in the continent of Africa, in Asia education, understanding of eye disease and societal norms could be addressed within these areas to be most effective in decreasing blindness in women that are most at risk for going blind. Future research could be within each of the rural communities and areas to determine what each population's unique needs and wants are in terms of eye care services and eye disease prevention methods [190, 191]. Health inequities could be addressed within eye research for Women living in Asia as it pertains to both societal structure and access to care. There are initiatives that have been started to address eye health within Asia. The Fred Hallows Foundation aims to determine the reasons for increased cataract dropout rates in Bangladesh. The initiatives results will help with health care planning in this population to best understand their eye care needs such as future intervention methods, in addition to educational material on the importance of obtaining cataracts surgery to prevent vision loss in this population [60, 61]. Health inequity needs to be addressed within eye research for Women living in Asia as it pertains to both societal structure and access to care.

2.2.3 Australia

Overall in the continent of Australia, women experience a higher burden of blindness compared to their male counterparts, as depicted in Table 2.1 [125, 137, 190]. This is also true in studies that observed solely Aboriginal populations of Australia [125]. Women in Australia also experience a higher prevalence of visual impairment compared to men based on a visual impairment gender ratio [190, 191]. Visual impairment prevalence is expected to decrease slightly, but Australian women are expected to still have a greater visual impairment due to loss of distance vision into 2020 [137].

Eye conditions are found more within the Aboriginal women of Australia and more common in Indigenous Australians as compared to non-indigenous Australians [192, 193]. This is because they are less likely to access and have access to eye care services and preventative methods for blindness such as surgery [192, 193]. This is due to geographic isolation, poorer access to transportation, and low-resources overall [194]. Research has also shown that Aboriginal and Torres Strait Islanders are more likely to have visual impairment as compared to other groups of Australians [195, 196]. This is because there is limited access to eye care providers in the rural area of Australia, so receiving eye examinations can be difficult [197]. Aboriginal and Torres Strait Islander women have a higher prevalence of vision loss as compared to Aboriginal and Torres Strait Islander men [196]. The 2016 National Eye Health Survey conducted in Australia found that Aboriginal and Torres Strait Islanders had a 1.4-increased risk of vision loss as compared to Aboriginal and Torres Strait Islanders men [196]. It is important that public health efforts learn the different eye health needs between each tribe or between each community to better address each individual population's unique care needs in terms of prevention of vision loss [12].

Telemedicine may be an option to combat access to eye care providers and eye examinations within rural areas of Australia. We will explore this intervention later within this review.

It has been reported that Australian women have a higher prevalence of glaucoma as compared to men [198, 199]. This is most likely because women live longer than men [199]. Women have higher rates of cataracts in men in Australia for those that are 50 years and older, again this is most likely due to the fact that women live longer than men in Australia [200, 201]. Furthermore, women are more likely to develop dry eye syndrome in Australia, which is similar to other industrialized countries around the globe [202]. McCarty et al. conducted a cross-sectional study in Melbourne, Australia to determine the prevalence of dry eye disease within the population. The study consisted of adults age 40 to 97 years of age. The study had 433 men participants and 493 women participants. The study demonstrated that women were more likely to have severe symptoms of dry eye disease as compared to Australian men [203]. This may be because women are more likely to develop an autoimmune disease, as well as hormonal changes during both pregnancy and menopause that can increase their risk for developing the disease and have more severe symptoms [50, 51].

The National Health and Medical Research Council in Australia recommends for diabetic pregnant women to receive an eye examination sometime within their first trimester and these results would determine how many other eye examinations they should receive during their pregnancy [204]. The National Health and Medical Research Council is similar to the American Academy of Ophthalmology in that they also recommend for those with type 2 diabetes, that they receive an examination at the time of diagnosis and yearly after [204].

Blindness and visual impairment for women remain a problem in Australia. Considerations for addressing women's eye health in Australia must be inclusive include Aboriginal and Torres Strait Islander women. It is vital that assumptions not be made for the eye care needs of the Aboriginal and Torres Strait Islander based on non-indigenous Australian women's vision needs. If this is done, non-indigenous women may only benefit from public health interventions, leaving Aboriginal and Torres Strait Islander women to continue to experience health disparities within vision care services.

2.2.4 Europe

European women are more likely to become blind or experience vision loss as compared to European men, as outlined in Table 2.1 of this review [190, 205, 206]. This may due to the fact that women live longer than men, but additional research should be done to further determine this [207]. Women in high-income Western Europe, Central Europe, and Eastern Europe experience greater visual impairment than men living in the same regions. All of these regions encompass Europe, so overall women in Europe experience greater visual impairment than men living in Europe [190, 208, 209]. The burden is greater for those in high-income Western Europe based on a visual impairment gender ratio [190, 208, 209]. High-income Western Europe includes countries such as Spain, Sweden, France, Germany, Norway, and the UK [210].

Women outlive men and this may be why the burden due to cataract is higher in women in Europe when observing the prevalence of overall eye disease in Europe [211, 212]. This is especially true in Poland, Finland, and the European elderly population [211, 212]. Overall blindness can also be found more substantially in women of the Netherlands as compared to their male counterparts per the meta-analysis conducted by Abou-Gareeb et al. [125] Nowak and Smigielski conducted a cross-sectional observational study including participants from central Poland [212]. The study included participants that were aged 35 years and older [212]. There were 465 men participants and 642 female participants within the study [212]. All of the study participants were of European ancestry [212]. They found

that there was a higher prevalence of cataracts amongst women, but it was associated with older age [212].

Cataract surgeries could help as a potential treatment for this eye impairment but the surgery is not always feasible. For instance, Poland is one of the European countries that have worse access to cataract procedures for treatment [213]. Though, it has been found that the incidence of cataract surgery has been increasing in Poland [214]. Further research efforts will need to determine if the higher incidence rate applies to women, or if men continue to benefit more greatly from the increased rate of cataract surgery [214]. Without the surgery as a treatment for cataract more women will continue to go blind. In high-resource areas, eye diseases such as glaucoma, diabetic retinopathy, and age-related macular degeneration are more common, while in low-resource areas cataracts contribute to a significant amount of vision impairment [215]. Women in high-income Western Europe may have a greater burden of visual impairment because of these diseases due to the aging population as compared to the overall burden of cataracts in the other regions of Europe. Glaucoma and ocular hypertension have also been observed to be higher within the population of women within the study, but again has been explained by the older age [212].

In Europe, there is an action group that raises awareness for vision and eye health needs for Europeans to prevent avoidable blindness and vision loss, as well as to make sure that the society in Europe is inclusive for those with low vision and irreversible blindness [216, 217]. This action group is the European Coalition for Vision [216, 217] The coalition hopes that more research pertaining to visual impairment in Europe will occur to provide information for policy and eye health decision-making [217]. This is an example of an initiative that can be beneficial to women's eye and vision health throughout Europe through health decision-making and policy that can impact the causes of visual impairment throughout the regions of Europe. We will explore further global initiatives later in this review that are working to improve women's eye and vision health in other parts of the world.

2.2.5 North America

Women overall experience both blindness and vision impairment more than men in the USA, as outlined in Table 2.1 of this review [218–220]. This also includes a wide range of eye diseases, including age-related macular degeneration (AMD) [36–38], glaucoma [29, 221], and cataract [222, 223]. Though women do not experience diabetic retinopathy more significantly than men in the USA, women almost have the same prevalence as men for the disease [223, 224]. Sex plays a role in the prevalence of these eye diseases within women, there are also noticeable differences in the prevalence of these diseases based on a women's age as well as their race and ethnicity identification. Current US statistics from 2010, presented by the National Eye Institute, can lend a hand to preventative care services through public health efforts to address eye diseases that affect women more significantly than men [225]. Table 2.2 depicts which eye diseases are more prevalent in women as compared to men by their racial and ethnic groups in the USA. The other category in Table 2.3 includes women who identify as Asian, Native American, and Pacific Islander who reside in the USA. Further surveillance of these eye diseases and factors should evaluate these populations individually to make the most significant public health impact to address visual impairment and eye disease that are imperative to these individual populations.

White women experience age-related macular degeneration (AMD) the most out of all the different race/ethnic groups identified by the national statistics presented by the National Eye Institute, followed by Black and Hispanic women respectively [36]. In terms of association of age with the disease prevalence for age-related macular degeneration, AMD increases with age in white women but stays about the same for the other racial/ethnic groups observed throughout their senior years [36]. The only group of women to have a higher

Table 2.3 Higher prevalence of eye health outcomes in women within the USA

Population	AMD	DR	Glaucoma	Cataract	Low Vision	Blindness	Hyperopia	Myopia	Vision impairment
White	X	X	X	X	X	X	X	X	X
Black	X	–	–	X	X	X	X	X	X
Hispanic	X	–	X	X	X	X	X	X	X
Other	X	–	X	X	X	X	X	X	X
Overall	X	–	X	X	X	X	X	X	X

X Women have greater prevalence than men; – Women do not have a greater prevalence than men

prevalence of this eye disease in comparison to men is white women, though in the overall population of people living in the USA, women and men have about the same prevalence for the disease [224]. The prevalence of Age-Related Macular Degeneration is highest amongst Hispanic women. The prevalence of diabetic retinopathy is highest in women who are aged 65–74 despite race and ethnicity [224]. In terms of Glaucoma, there is a higher prevalence of cases of glaucoma in White, Hispanic, and other women, while African-American men and women have about the same number of cases [221]. Specifically, open-angle glaucoma is the most common form of glaucoma [221]. All women in the USA, despite what their racial/ethnic background, have more prevalent cases of cataracts compared to men, with white women having the highest prevalence rates [222, 223]. Moreover, the overall number of prevalent cases of cataract increases with age no matter which racial and ethnic group [222].

In addition to eye disease, women in the USA also experience greater factors that can also compromise their ability to see at a higher prevalence than men. One of these factors is Myopia [39, 40]. Myopia prevalent cases were more significant in women for all racial and ethnic backgrounds [40]. White women had the most considerable number of prevalent cases, followed by Black women. Hyperopia is another refractive error found with a higher prevalence in women in the USA [131, 218, 226]. White women have the highest prevalence rate of Hyperopia [131]. Age seems to increase the prevalence rate of Hyperopia no matter what a women's race and ethnicity identification are [131]. In terms of overall vision impairment, women in the USA experience it the most [190, 218, 227]. This is true for all age and racial and ethnic categories [227]. The prevalence of cases of visual impairment increases with age [227].

There are recommendations for comprehensive eye examinations depending on age, risk factors, and disease status. The American Academy of Ophthalmology (AAO) recommends that everyone receive a comprehensive eye examination at the age of 40 years old. For those aged 40 to 54 years of age with no risk factors for eye disease are recommended to get an eye examination every 2 to 4 years [228, 229]. Those with no risk factors for eye disease aged 55 to 64 years of age should receive a comprehensive eye examination every 1 to 3 years [228, 229]. Those who are 65 years of age and older and who have no risk factors for eye disease should receive a comprehensive eye examination every 1 to 2 years [228, 229]. Women who are pregnant with diabetes are recommended to get eye examinations before they become pregnant as well as early in the first trimester. Those with higher risk factors for developing eye diseases, such as family history, medical history, ocular history, age, or race should receive comprehensive eye examinations more frequently [228, 229]. An example of this is that those with diabetes mellitus type 2 should be examined first when they are diagnosed and then every year after [228, 229].

Each community may need their own plan to address women's eye health because of the differing needs even within the community. An example of this would be the Native American population within the USA. Native American women have high rates of type 2 diabetes and hypertension [230]. These chronic diseases cause lasting effects on the eye such as the manifestation of diabetic retinopathy and hypertensive retinopathy [12]. Native American reservations can be in geographically isolated locations, so

access to quality preventative eye care services may be difficult to obtain. Another community that may need different eye care needs then the overall population of women living in the USA is Appalachian women. Appalachian women also live in a geographically isolated area of the USA of America. Receiving preventative eye care can be difficult for those that live in geographically isolated areas. In addition, those living in the Appalachian region have unique barriers to healthcare due to socioeconomic status, culture, and substance abuse [231]. Those that live in the Appalachian region have higher rates of diabetes as compared to the general US population [231]. In addition, those living in this region have higher mortality rates from diabetes as compared to the overall US population [232]. This means that those living in this region may have uncontrolled diabetes which can negatively affect the eyes. Uncontrolled Diabetes can manifest in the eye as diabetic retinopathy causing permanent vision loss. Public Health interventions should focus on planning and delivery of quality, relevant care to have the greatest impact within the Native American and Appalachia population of women living in the USA. Telemedicine may be an approach to address the issue of access for Native American and Appalachia women in geographically isolated locations. We will further explore telemedicine later in this review. Research that aims to address improvement in health within these underserved populations should focus on health disparities that affect these populations to improve eye health for women.

The National Eye Institute supports an initiative conducted by Harvard University that has established Women's Eye Health (WEH) [58, 59]. The WEH's mission focuses on education that is relevant to eye diseases that are most commonly found more prevalent within women, occur more in women due to older age and eye diseases that increase based on both environmental and lifestyle factors that affect women [59]. In addition, partnerships have been established to better understand eye health of women, such as the partnership between Well-Intergraded Screenings and Evaluation for Women across the Nation (WISEWOMEN) and Prevent Blindness North Carolina [233]. WISEWOMEN's focus is on providing services to women to promote lifestyles that will keep their hearts healthy [233, 234]. Moreover, they focus on providing information to women so that they can better reduce their risk of heart disease and stroke [234]. Prevent Blindness North Carolina is a nonprofit health agency [233, 235]. They offer service programs related to the prevention of blindness through publications, safety, screenings, education, and information [235]. This partnership gave vision screenings to underserved women ages 40–65 years old. Most of the women were from low socioeconomic status households. The program was able to obtain responses to risk assessment questions within their vision-screening program. This information was ready to be shared with county coordinators of WISEWOMEN. This collaboration allowed for information growth and vision screenings for women within these communities [233]. Future research collaborations across prevention programs could implement a similar model to determine risk assessment for women in other regions. Other partnerships focus on specific eye diseases such as the partnership between the National Eye Institute, the Office on Women's Health and the Society for Women's Health Research's targeted approach on addressing dry eye syndrome in women [236]. This initiative provides education to increase awareness of the problem of dry eye disease for women. The action put into place by this initiative also provides clinicians with diagnostic and treatment methods [236].

Canadian women have a higher prevalence of blindness as compared to Canadian men [137, 190]. Canadian women have also been found to be more likely to have visual impairment compared to Canadian men [190]. Maberly et al. conducted a study in Canada and found that females had higher degrees of blindness as compared to men [237]. The 2012 Canadian Survey on Disability (CSD) found that seeing disability for Canadian females was greater than for men overall [238]. This was especially true for women that were between the ages of 24 to 44 and 65 to 74 years of age [238]. This survey was conducted with participants that were aged 15 years and older and had a vision disability that affected

their daily tasks [238]. Stevens et al. reported that after controlling for age women had a greater prevalence of blindness as compared to men in all regions of the world [190]. Where the difference between men and women was the greatest was within regions of high-income. Canada is considered a high-resource country [239]. Women in high-income regions were found to have a 1.5 higher prevalence of blindness as compared to men [190]. In high-income area's biological and genetic factors could contribute to this greater difference between men and women. The gender difference between men and women may be lower in countries with low-resources because men may face more similar barriers to women within these countries.

Women in Mexico have a higher rate of blindness than men living in Mexico [137, 190, 240]. Moreover, Women in Mexico also have a higher burden of visual impairment as compared to men in Mexico [137, 190]. Polack et al. reported that women had a higher prevalence of blindness than men. This study was a cross-sectional population-based study in Chiapas, Mexico with 2864 participants [240]. In Mexico, women have a higher prevalence of cataracts than men, which tends to increase with age within this population [241]. Also, dry eye disease related to ocular surface damage has a higher burden in women who are of older age [242, 243]. This occurs because of hormonal changes for women [242, 243]. In addition, they are also more likely to have an autoimmune disease causing dry eye syndrome [242, 243]. Rodriguez-Garcia et al. reported on the profile of patients that were residing in Mexico that had damage to the ocular surface due to dry eye syndrome [242]. It was found that those that were affected by this were women of older age [242]. This population-based prospective cross-control study included 1543 women and 1182 men of all ages [242]. Mexico was the first of America's to wipe out trachoma, which is still an epidemic and cause of blindness for many women across the world including Africa and Asia which we have previously discussed in this review [244]. Public health, which pertains to eye health and vision loss in North America, could focus on access and resources to eye care within the population. Each country should have a public health initiative to address their eye care needs, as well as within their individual communities [12].

2.2.6 South America

Women in South America experience a greater burden of blindness as compared to men in South America, as outlined in Table 2.1 of this review [137, 190]. Limburg reported that women of Paraguay, Peru, Argentina, Cuba, Venezuela, and Guatemala have a greater prevalence of bilateral blindness for those aged 50 years and older [245]. These results were concluded from population-based prevalence surveys within Latin countries in those that were age 50 years and older. The exact numbers of participants who were male and female were not specified [245]. In the Sao Paulo Eye study, researchers found that older women in Brazil had a higher presence of blindness compared to Brazilian men, but the education level of the women explained this [246]. Within the study, there were 1834 males and 2136 females that were eligible and 1542 males and 2136 females that were examined within the study. The study only consisted of participants that were age 50 years and older [246]. The study was conducted in a cross-sectional manner to randomly obtain study participants [246]. The study was used clustering sampling and conducted door-to-door surveys and ocular examinations [246]. Cross-sectional studies are important for women's eye health research in order to determine the prevalence of a disease in a specific area so further research can be conducted, as well as tailored intervention methods that are important to the specific area that was included within the research study. Women in South America also experience a greater prevalence of visual impairment [137, 190]. Women of Tropical Latin America have a slightly higher prevalence of visual impair-

ment compared to men. Andean Latin America, Central Latin America, High-Income Southern Latin America, and the Caribbean all have a greater prevalence of visual impairment compared to men [137, 190]. We will now explore factors that may be contributing to the higher prevalence of these eye diseases that come in the form of biological and social factors that affect eye health and vision within women around the world, which are outlined in Figs. 2.2 and 2.3 of this review.

In Brazil, women have higher rates of dry eye syndrome compared to men. This can help to explain that women are more likely to experience dry eye syndrome as compared to men [247]. A study by Limburg et al. found that countries in South America including Brazil, Venezuela, Guatemala, Argentina, Peru, and Paraguay has higher blindness and low vision from cataracts is within their population of women as compared to men [248]. This study used data from population-based surveys that observed the prevalence of cataracts within Latin America [248]. The surveys were conducted between 1999 and 2006 [248]. The surveys included individuals that were 50 years and older [248]. Future research should aim to provide the number of male and female participants to provide a thorough presentation of the demographics of the population of women that may be affected the most within these countries. Pongo Águila et al. conducted a study in a semirural area in Northern Peru and found that women experienced a higher prevalence of blindness from cataract and other causes compared to men [249]. The study areas were two cities in Peru, Piura, and Tumbes [249]. There were a total of 2221 men and 2561 females [249]. This study used systematic cluster sampling for participants 50 years and older. The study collected information on general demographics, visual acuity test results, lens examination results as well as cataract surgery information [249]. In low-resource countries within South America, eye care can be challenging to access due to the cost of care, transportation barriers to reaching the eye care services, lack of access to information pertaining to both eye health and disease, fear of a negative outcome in terms of eye care treatment and the perceived value of eye care treatment [126]. These barriers may impact women more greatly, due to societal views of women and their health needs in some of the South American countries [250, 251].

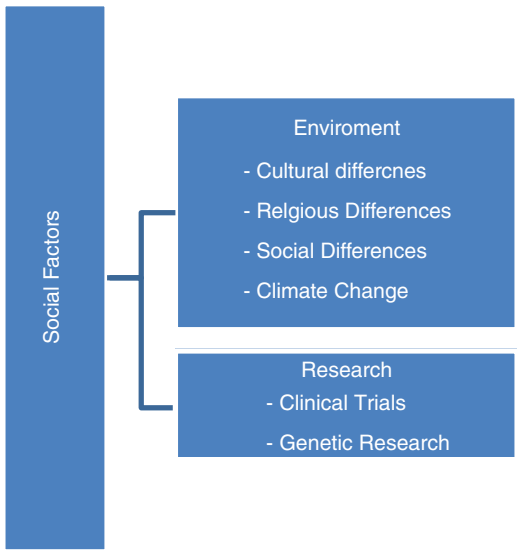

Fig. 2.2 Social factors for women's eye health

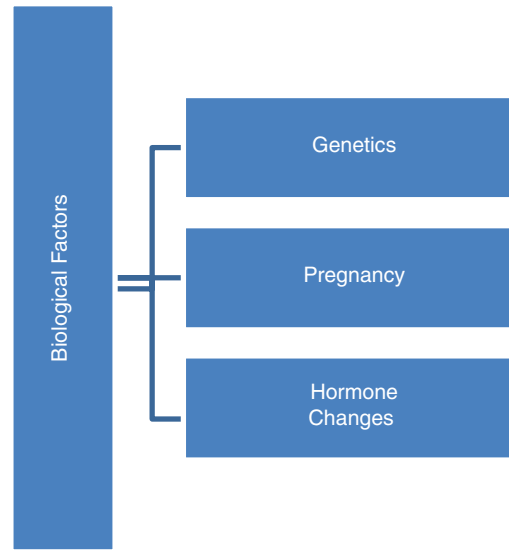

Fig. 2.3 Biological factors for women's eye health

2.3 Aging and Eye Disease in Women

Vision impairment is a public health problem for elderly patients around the world [252–254]. Overall, women outlive men, and because women live longer it contributes to the overall effect of eye disease on women across the globe [16, 18, 55]. There are a higher number of women within the older age groups in the overall global population, which is associated with a higher risk of blinding eye disease [95]. Health care costs for women over their lifetime are more significant than men, most likely because women live longer than men [255, 256]. Health care costs are strongly dependent on age because as someone ages they tend to need more medical care [255, 257]. Due to the progression of the eye care and vision needs of women as they grow into older age their health care costs can increase for eye care. This is in terms of routine eye care, medical devices such as glasses as well as surgery to prevent blindness such as the case of cataracts. It is crucial that as women age, they continue to monitor their eye health and vision needs by receiving annual routine eye examinations to detect eye diseases associated with a higher risk of eye disease and visual impairment from older age [258–260]. A women's quality of life can diminish as they age because of eye diseases and c visual impairments [261, 262]. Visual impairments are associated with physical and mental comorbidities in older adults [263, 264]. This can be seen in the case of visual impairments due to refractive errors. Refractive errors, which more greatly affect women in older age, are associated with lower quality of life due to depression, falls, and fractures [265, 266]. Due to these qualities of life factors, women may not be able to do their routine activities or may depend on others for help and care. Dependence on others may be more difficult for women living in certain parts of the world for social and cultural reasons. Social and culture norms may limit a women's ability to get help while living with vision impairment because their needs may not be help to the same standard as men. We will further explore social and cultural norms as a barrier to eye health in women, later in this review. Routine eye examinations are essential during the senior years of life for women to continue to be able to do their daily activities, as well as manage any eye diseases that they may develop as they get older.

2.4 Heritable Ocular Conditions

Genetic factors may also be a contributor to the higher prevalence of blindness and visual impairment for women across all parts of the world. Women like men may have a genetic risk for particular systemic diseases that also involve the eye resulting in visual disability. An example of this would be that women are at a higher risk for autoimmune disease because the associated immune genes are associated with the X chromosomes [53, 54]. Certain Autoimmune diseases affect women more than men. These autoimmune diseases include Lupus, Sjögren's syndrome, and Grave's Disease [267–270]. Autoimmune diseases can have negative impacts on vision. Another autoimmune disease that affects women more often than men is, Neuromyelitis Optica (NMO). NMO is a central nervous system autoimmune disease that affects the optic nerves and spinal cord the most [271]. Furthermore, women are nine times more likely to reoccurring episodes as compared to men [272, 273]. Oculofaciocardiodental syndrome is a rare condition that causes eye abnormalities and is only found in women due to its X-linked inheritance pattern [274–278]. The syndrome causes congenital cataracts and those with the syndrome are at a greater risk for glaucoma [277, 278]. A case report by Martinho et al. describes a case of Oculofaciocardiodental syndrome in a female aged 26 years old [277]. The patient had a diagnosis of cataracts in both of her eyes and her eyes were observed to be very deep set [277]. Future research will need to focus on equal representation within genetic research for women to benefit from personalized medicine pertaining to ocular disease that may have genetic associations.

2.5 Pregnancy and the Eye

Women may become pregnant sometime in their life, which can cause temporary changes to their vision but in some cases cause life-long damage to their vision. Pregnancy can affect both the physiology and pathology of the eye [49, 279, 280]. This can cause changes in both the eye and vision [49, 279, 280]. These changes during pregnancy can come from differing hormone levels, the amount of excess water in the body, variation in blood pressure, and increased blood volume in the body [49]. These changes could potentially lead to blurry vision, dry eyes, the shape of the cornea or cause nearsightedness [49, 281]. During pregnancy visual disturbances is a common complaint about many women [282, 283]. Mehdizadehkashi et al. conducted a cross-sectional study of 107 pregnant women aged 20 to 39 years of age in Iran [282]. The study observed myopic and hyperopic visual changes during pregnancy that decreased distant acuity vision and near vision acuity [282]. The participants' vision returned to normal after their pregnancy [282].

Women may learn that their vision will change during their pregnancy and choose to not visit their primary eye care provider for their dilated annual routine eye examination until after their pregnancy is over. This comes with risks because if they do not obtain their regular eye care it can cause certain eye diseases that may form from pregnancy-induced conditions, such as hypertension retinopathy and diabetic retinopathy, to go undetected [284–286]. Hypertension, if it goes untreated can be a risk factor for hypertensive retinopathy, and similarly, diabetes can be a risk factor for diabetic retinopathy [73, 287–289]. Retinopathy risk increases if a woman experiences preeclampsia or eclampsia during their pregnancy [290, 291]. Preeclampsia can also lead to permanent changes within the eye that include optic atrophy, optic neuritis, and retinal artery and vein occlusion, though this happens rarely [290, 292]. Women who have hemolysis, elevated liver enzymes, low platelets syndrome are more likely to have bilateral retinal detachment which can occur during pregnancy, though this is rare [293–295]. Exudative retinal detachment can occur in pregnant women with disseminated intravascular coagulation and thrombotic thrombocytopenic purpura [295, 296]. The recommendation for women who are pregnant is to continue to get their annual routine eye examinations, especially if they already have severe eye diseases such as diabetic or hypertensive retinopathy [297–299]. In some cases, women may need to have their vision checked more often if they have a preexisting condition because pregnancy can worsen their preexisting eye diseases [300, 301].

Pregnancy is a gender-specific factor that needs to be considered for visual genetic conditions. Pregnancy is a life event that only affects women. There are genetic conditions that pertain to the eye that can negatively impact a women's health during pregnancy. For example, women who have the genetic condition Pseudoxanthoma elasticum (PXE), which can manifest within the eyes, have a higher risk for gastrointestinal during pregnancy [302, 303]. Women who have Marfan syndrome, a genetic ocular condition, have a higher risk for aortic dissection during pregnancy [304–306]. Women also have an anatomical predisposition for primary angle closure glaucoma which can lead to blindness if not properly treated [29, 307–309]. Women who have glaucoma will need to communicate with their eye care provider that they are pregnant because pregnancy can affect a women's intraocular pressure, usually lowering the intraocular pressure [279, 281, 301]. Their primary eye care provider may alter their glaucoma medication according to their needs during pregnancy. Pregnancy is not the only biological difference between men and women that can cause changes to their vision. We will next discuss hormonal changes that women experience later in life that can have an effect on their vision.

2.6 The Effects of the Stages of Menopause and the Eye

Many women begin transitioning to menopause between their mid to late forties [310–312]. For most women, menopause usually occurs between the ages of 45 to 58 years old [311, 312]. Hormonal changes during these times and post-menopause can cause both eye disease and vision changes for women across all regions of the world [50, 313, 314]. Symptoms of menopause vary around the world and in India diminished vision is said to be an indication of menopause [315]. These changes for eye health and vision are due to lower sex hormone levels during the times of menopause and post-menopause.

A woman's eyesight may fluctuate during menopause. This usually causes less nearsightedness and may result in their need for glasses to help with reading. In terms of eye disease, dry eye disease is common in menopausal women because of the hormone changes that are occurring [50, 51]. The hormone estrogen and the hormone androgen that help to keep the eye lubricated are found to be at decreased levels during menopause causing women to experience the dryness, redness, itching, and burning symptoms of dry eye disease [50, 51]. Postmenopausal women also experience a more significant burden of dry eye disease as compared to younger women [50, 52]. Olaniyan et al. conducted a cross-sectional community-based study in South-West Nigeria to determine both the prevalence and risk factors for dry eye syndrome [316]. The study was conducted in adults aged 40 years and older using both questionnaires and eye examinations [316]. The study observed that menopausal status was statistically significantly associated with dry eye syndrome [316]. Postmenopausal women may find that it is more difficult to wear their contact lenses during this time due to dry eye disease and the changing shape of their cornea [317, 318]. In addition, dry eye disease can affect their quality of life because they may no longer be able to do their day-to-day activities without eye fatigue or strain such as reading, driving, or watching television [319–321]. These hormonal changes are similar to what is seen during the menstrual cycle for a woman [51, 322, 323]. Hormone levels change throughout a women's menstrual cycle which can affect the cornea by changes in surface dryness and tear production [51, 322, 323]. Further research surrounding dry eye disease for women, should account for both menstrual cycle and menopause state as a variable, as the menstrual cycle can have a role in if the patient is experiencing dry eye symptoms. Postmenopausal women are also at increased risk for the burden of eye disease. Sex hormone deficiency in postmenopausal women can increase their intraocular pressure, which in turn can increase their risk for developing glaucoma, though further research is needed to improve the association of this risk [324, 325]. Research has shown that women who experience menopause before 43–45 years of age have an increased risk for primary open-angle glaucoma (POAG) [70, 74, 107].

Treatment of menopausal symptoms can affect women's eye health and vision. In some cases, women may choose to treat menopausal symptoms using hormone replacement therapy. The US Food and Drug Administration has listed vision loss due to blood clots in the eye as a severe side effect from some of the treatments, and jaundice also can occur from the treatment which can cause the whites of one's eyes to turn yellow [75, 79]. Hormone replacement therapy can also cause dry eye disease in some cases, though it is still debated depending on the length of time and the type of hormone replacement therapy that is used [50, 80]. As we have explored, biological differences between men and women can cause variations in vision and eye health such as in the case of aging, pregnancy, and menopause. These biological sex differences may be unavoidable, but they are essential when assessing the eye care needs of women around the world. We will now explore social factors that may contribute to differences in eye health and visual impairment in women based on gender to precisely identify the different cultural and societal roles that women may endure that men do not [15].

2.7 Climate Change and Women Eye Health

Climate change can impact vision from air pollutants, increased ultraviolet (UV) exposure as well as arid land [81]. Multiple parts of the eyes are exposed to these factors such as the cornea, eye lid, sclera, and the lens of the eye. Increased UV light exposure is a risk factor for cortical cataracts, while an increase in arid land can increase dry eye syndrome due to the drier air conditions [81]. Furthermore, climate change has also increased the air pollutants that can cause both eye discomfort due to dry eye and irritated eyes [81, 82]. Climate change not only affects air quality, but also can affect food security because it can affect crops. Climate change can also diminish water quality and increase people's exposure to both viral and bacterial pathogens [85]. Women are disproportionally burdened by climate change as compared to men. An example of this can be observed in a study conducted by Cil and Cameron 2017 in terms of climate change and pregnancy. The study used monthly panel data from the US Natality Files (1989–2008) for more than 3000 counties within the USA [86]. They observed that heatwaves increased the risk for pregnancy-associated health conditions such as eclampsia, pregnancy-associated hypertension, incompetent cervix, and uterine bleeding [86]. The Commission on the Status of Women has made this a priority [87–93, 326–330]. Natural disasters burden low-resource communities more than communities that are not [88–90]. In regions around the world where women's eye health may be more negatively impacted by social and cultural contributing factors such as low-resource areas of Africa and Asia, climate change can have a worse burden. Women make up more of the population that is living below the poverty line and therefore are more likely to be affected by climate change and natural disasters [91].

Risk factors that we have discussed throughout this review have the potential to be amplified due to climate change. Women who reside in arid land areas not only have to deal with their biological susceptibility of developing a dry eye disease, but environmental factors of the drier air amplify the dry eye. Women in low-resource areas in Africa [35, 145, 168] and Asia [92, 93, 326] are already at a greater risk for developing cataracts; increased exposure to UV light may worsen this risk. In Table 2.2 it is observed that women have higher rates of cataracts in Australia as well. This could be due to their UV exposure rates as well as social factors that impact their health. UV exposure in Australia is high due to their geographical location [327]. Regions in high-resource areas of Australia could be less burdened by cataracts due to UV exposure because they have access to cataract surgery, while those in low-resource regions may be more significantly burdened by cataracts due to UV exposure because of their limited access to cataract surgery.

If women are unable to access nutritious foods they could develop systemic diseases such as diabetes and hypertension which could manifest themselves in the eyes as diabetic and hypertensive retinopathy [12]. Climate change has the ability to be a risk amplifier for these diseases which could cause blinding retinal diseases. In addition, the Risk of Trachoma, which as previously explored is due to not having proper water source accessibility, can also be amplified. Furthermore, because climate change can increase bacterial illnesses we observe a greater risk for River Blindness for women living in areas with low-resources that are primarily affected such as rural parts of Africa. Climate change may be considered in the evaluation of the implementation of interventions to improve women's eye health around the globe, while consideration of gender has recently implemented within both climate change and policy, the progress has been slow to decrease gender-based health disparities [90].

2.8 Approaches to Interventions

2.8.1 Telemedicine

Telemedicine, specifically teleophthalmology for the use of eye care has been becoming more prevalent increase access to eye care for those in rural and underserved areas [328–330]. Telemedicine allows for healthcare delivery to those that may not otherwise be able to have access for diagnosis, treatment, and preventive health purposes [331]. Telemedicine can be used for detecting, screening, and diagnosis of retinal eye disease such as diabetic retinopathy [328–330]. Telemedicine also has the potential to provide information for research that would include participants who may otherwise not participate in scientific research and are not well represented within previous research studies [331]. Telemedicine could potentially inform researchers of specific eye care needs of the underserved communities. This can help to influence both public health services and public health policy. We will discuss research and women's eye health in the next section. Research surrounding telemedicine options for eye care should continue. In addition, further funding should be allocated to these efforts for women around the globe who may not otherwise be able to receive eye care to prevent visual impairment.

2.8.2 Empowerment

An important aspect to addressing these barriers for women as they pertain to social and cultural norms is women empowerment. Promoting women's empowerment can improve health outcomes, including eye health. There are varying forms of empowerment that could be implemented to improve women's health around the globe including financial, community, and social empowerment. Financial empowerment of women can help to improve their economic stability and provide them with an increase in resources to improve their overall health [332]. A study by Roy and Chaudhuri conducted in India with a total of 34,086 individuals, 49.4% woman and 50.6% women explored the issue of financial empowerment [174]. They found that older women in India were more likely to have poorer health and utilize healthcare less often, compared to men in India, due to both socioeconomic status and financial empowerment [174]. These same approaches should be further explored in terms of eye care utilization and financial empowerment for women in low-resource areas. In low-resource areas, women's health needs may not be considered a financial priority within their households. Financial priority for health needs is for men as the head of the household followed by the children with sons having more of a priority. By researching mechanisms to financially empower women, this can lead to improved health outcomes because they will be able to financially make their health a priority because of the new gained resources. These gained resources can include the ability to travel to their eye care provider as well as financially afford glasses and eye care services. Social empowerment addresses the improvement of women's self-confidence, self-identity, and social belonging [333]. Socially empowering women can help them to make their health, including their eye health, a priority. Women can be thought of as a community and as such can achieve community empowerment together. The World Health Organization states that when community empowerment is successful that communities are able to have improved control over their life [334]. This action of community empowerment can also help to increase their community involvement to help other women, as well as children, seek out necessary eye care to prevent visual impairment. It also can bring about both social and political change to improve both overall health and eye health. It is important that women around the globe be empowered through all aspects of empowerment (financial, community, and socially) to have a lasting change on their health, which can also make a lasting impact on their eye health. Each individual community will have different empowerment needs and should be individually evaluated.

Globally, women's social status within their community and within their families and households can impact their ability to gain access to eye care services to prevent blindness and visual impairment [92, 127]. These barriers to eye care

can come in many combinations of factors and make accessing eye care an unattainable goal for women globally, leading to preventable blindness. Barriers to eye care for women cause health disparities that must be addressed to achieve health equity within the field of eye and vision care. Telemedicine may help to increase access to eye care health for women that are not able to reach eye care in a standard medical setting. Research that focuses on women's eye health needs and also that address these barriers is essential to improving eye care for women around the globe.

2.8.3 Research

There are differences between clinical decisions made for men and women [45, 335]. These clinical decisions can be better understood from clinical and basic research, which women have been less represented throughout history [45, 46]. As we have previously mentioned, both biological and social factors differ between men and women which can be better understood by medical research. Women may benefit less from advancements that are gained from medical research that focuses on eye health and visual impairment because they are less represented within clinical research studies as compared to men. When women are less described in clinical research, they may also benefit less than men as it pertains to advancements in personalized medicine, which is a tailored approach of treatment for an individual patient based on their characteristics [336, 337]. Moreover, risk factors for health outcomes may go undetected or underpowered for women if research studies do not include women or if women only make up a small proportion of the total research study population. Findings from medical research studies that only included men within their study populations have been applied to women in the past, which can cause different treatment success rates between men and women [338].

Observing eye diseases that affect women the most, eye and vision care needs of women, as well as the social differences between men and women throughout this review, has shown that women have unique requirements for the maintenance of their eye health and vision. Medications for treatments of ailments can have different effects on people and because of this may differ between men and women. It is essential to understand these differences that may differ by gender in terms of medications used for the treatment of eye disease [45, 339]. Furthermore, symptoms of eye disease can vary between men and women. This understanding furthers the need to have equal representation throughout medical research. These differences present themselves as continued areas of focus for women's health research. When researchers are considering the design of the path they will take to execute their research studies, they need to be aware of differences in enrollment and retention of women in research studies [45, 47, 48]. Moreover, women are less likely to enroll in clinical research studies or know about them so recruitment factors pertaining to women must also be considered when designing a research study [45, 340]. These challenges in achieving the necessary recruitment and participation of women within their studies should be addressed by researchers rather than avoided, for them to be successful in achieving women's participation in their research.

Efforts have been made by the National Institute of Health to improve women's involvement in clinical trials [45, 341, 342]. The Office of Research for Women's Health works to address this problem within clinical research by working in partnerships to promote women's participation within clinical research, as well as underserved populations who have historically been underrepresented in clinical research [62, 343, 344]. The Office of Research for Women's Health also focuses on supporting and enhancing research that focuses on health issues that have the greatest impact on women [62]. Moreover, they work to achieve women representation in biomedical careers [62]. In terms of the National Institute of Health-funded clinical trials, more than half of the participants are women [341]. Furthermore, there is continuous growth in the number of research studies that are accounting for sex within research study findings and reports [343]. Though there have been advancements in the representation of women in clinical research it is essential that efforts continue to ensure that

women continue to be a part of research to benefit their specific health needs and outcomes.

2.8.4 Initiatives

The understanding for the need to address women's eye health has begun to come to the forefront over time. New efforts to tackle this public health issue are underway. We will explore some of these initiatives, but it is essential to know that this is not an exhaustive list of everything that is or can be done to improve eye health and address visual impairment for women. First, in terms of health observances, April is Women's Eye Health and Safety month [56, 57]. Observing Women's Eye Health and Safety month, can bring the challenges that women face in terms of keeping their eye's healthy and their vision clear to the forefront. Furthermore, acknowledging this month can help to educate a greater number of women on the importance of keeping of making their eye health a priority in living a healthy lifestyle. Initiatives can lead to an increase in advocacy for women's eye health. Continuation of advocacy improves equal access to eye care for women across the world. It is vital that advocacy takes place at all levels: the national, district, and community levels. It is crucial that none of these levels for advocating go under looked to achieve improvement in women's eye health [17]. Each population will need a different set of initiatives. If women's eye health needs are only addressed at the national level, smaller community-level needs can go unaddressed, causing continuous health inequity for women within these populations.

2.9 Conclusion

In all of the organ systems in the body, including the eye, there are differences between men and women in terms of types of disease, the prevalence of the disease, risk factors, and characterizations of disease [345]. Much of the burden from both visual impairment and blindness globally comes from inequitable eye care services [346]. Women living in low-resource countries or communities' may have less access to preventative measures to maintain health [12]. Understanding of blinding eye conditions and disease in women is vital for not each individual continent but country and population. Furthermore, understanding the unique factors of different cultures and societies for women within both countries and continents should further be explored. Not all women are the same, and findings from one group of women may not apply to women living in another country, continent, or population. An example of this would be findings from a study focusing on eye health need in Africa may not be applicable to women in the USA. Women in Africa may need care that is directly focused on Trachoma [114–117] and River Blindness [159, 161] which is not a burdening problem for Women in the USA. Biological factors as outlined in Fig. 2.3 and social factors as outlined in Fig. 2.2, as well as combinations thereof, should all be considered when addressing eye health and visual impairment in women around the world.

Though eye disease and condition prevalence may be marginally greater in women, these differences can add up over time. Equal representation within genetic and epidemiologic research is needed for women to benefit from personalized medicine pertaining to ocular disease. The field is continuing to expand so now is the time for researchers to ensure that women are participants within their studies, and to actively implement strategies to improve women's willingness to participate. When health disparities within eye health and visual impairment continue to occur for women, over time the differences in the burden of blindness can become more divided between men and women. Though it has been observed in women in low-resource countries that social factors may play a role in eye disease, care, and conditions this does not mean that they do not exist in countries with greater resources. Individual evaluations of concerns about gender differences in vision loss and blindness need to be conducted at the community, country, region, continent, and global level. All evaluations need to both consider and report sex within their evaluation reports when determining their next steps to address the problem.

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Appendix

Supplemental Tables

Supplemental Table 1 Initiatives to improving women's eye health

Initiative name	Goals	Website
National Women's Eye Health & Safety Month	Brings awareness to the challenges that women face in terms of keeping their eye's healthy and their vision clear.	https://www.preventblindness.org/womens-eye-health-and-safety-awareness-month
Women's Eye Health (WEH)	Focuses on education relevant to eye diseases that are most commonly found more prevalent in women, occur more in women because more women outlive men and eye diseases that are increased based on both environmental and lifestyle factors.	https://nei.nih.gov/content/womens-eye-health
Fred Hallows Foundation	Work towards closing the gender gap within avoidable blindness.	https://www.hollows.org/shesees
Seva Foundation	Work towards having gender equity within eye care to limit barriers to access to eye care.	https://www.seva.org/site/SPageNavigator/Seva_Vision_for_Gender_Equality_in_Eye_Care.html
Women in Eye and Vision Research (WEAVR)	Work towards development and strengthening of women who are pursuing careers in the visual sciences.	https://www.arvo.org/arvo-foundation/what-we-fund/women-in-eye-and-vision-research/

Supplemental Table 2 Additional resources on women's eye health around the world

Resource name	Objective	Website
Universal eye health: a global action plan 2014–2019	Universal accesses to health care, including eye care, as well as gender equity.	https://www.who.int/blindness/AP2014_19_English.pdf?ua=1
Eye Health for Women and Girls: A guide to gender-responsive eye health programming	Supplement for current knowledge that has been successful in addressing the gender gap in eye health programing.	https://www.iapb.org/wp-content/uploads/2017-Guide-to-eye-health-for-women-and-girls.pdf
United for Sight Module 3: Accessing Medical Care: Unique Barriers for Women	Community eye course to increase understanding of patient barriers to eye care to achieve quality eye care for all.	http://www.uniteforsight.org/community-eye-health-course/module2b
Office of Research on Women's Health	Promoting research focused on women's health.	https://orwh.od.nih.gov/
International Agency for the Prevention of Blindness	A tool to aid in decreasing unnecessary blindness and visual impairment.	https://atlas.iapb.org/
Pan American Health Organization (PAHO) and World Health Organization (WHO). Gender and Health Advocacy Kits:	Provide information on current issues on gender and health	http://www1.paho.org/english/hdp/hdw/advocacykits.htm

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3 Establishing the Chinese Arm of GEGC

Zui Tao, Shiying Li, Jiayun Ren, Xiaohong Meng, Yong Liu, and Zhengqin Yin

Abstract

Hereditary eye diseases have gradually become the main cause of refractory or incurable binocular blindness, a large number of which have no effective treatment. In order to further popularize and promote the ability of diagnosis, treatment, and prevention of hereditary eye diseases in China, the Chinese Eye Genetics Consortium (CEGC) is established, which is attached to the Global Eye Genetics Consortium (GEGC). On June 28, 2018, "The Founding Conference the Chinese Arm of GEGC" was grandly held in Chongqing, China. CEGC includes the Chinese eye genetic disease research collective, the Chinese eye genetic disease experts collective, and the Chinese eye genetic disease prevention and treatment collective. The consortium aims to carry out research on the prevention and treatment of eye genetic diseases, formulate guidelines and consensus for clinical diagnosis of eye genetic diseases, support and cultivate professional talents, and promote international exchanges and cooperation.

Keywords

Hereditary eye diseases · Establishing CEGC · GEGC

The Chinese Eye Genetics Consortium (CEGC), also known as the Chinese branch of the Global Eye Genetics Consortium (GEGC), includes the Chinese eye genetic disease research collective, the Chinese eye genetic disease experts collective, and the Chinese eye genetic disease prevention and treatment collective. CEGC is a professional academic alliance composed of experts engaged in clinical diagnosis, treatment, and prevention of eye genetic diseases. The consortium aims to carry out research on the prevention and treatment of eye genetic diseases, formulate guidelines and consensus for clinical diagnosis of eye genetic diseases, support and cultivate professional talents, and promote international exchanges and cooperation. CEGC is under the leadership of the council, attached to the GEGC, and participates in the academic exchanges and cooperation of GEGC. In addition, its form and rules are decided by the council of CEGC.

Background: Hereditary eye diseases are the main components of hereditary diseases. In clinical practice, 90% of diseases are related to genetic factors, of which 20%–30% are hereditary eye diseases or multi-organ diseases containing eye abnormalities [1]. As the living standards

Z. Tao · S. Li (✉) · J. Ren · X. Meng · Y. Liu · Z. Yin
Southwest Hospital/Southwest Eye Hospital, Army Medical University (Third Military Medical University), Chongqing, China

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improved, people put forward higher requirements for vision. With the development of medical technology, some common blinding diseases, for instance, cataract (51% of blind people in the world, WHO, 2010) and trachoma (3% of blind people in the world, WHO, 2010) can be effectively controlled or even cured by surgery or drugs. But a large number of inherited eye diseases have no effective treatment. Hereditary eye diseases have gradually become the main cause of refractory or incurable binocular blindness, which is the focus of present and future prevention and treatment of blindness [2, 3]. Therefore, GEGC arose in response to engaged in clinical diagnosis, treatment, and prevention of eye genetic diseases.

The number of visually impaired people in China is 755.11 million in 2010. Among them, 67.26 million people have low vision, while 8.25 million are blind (WHO 2010). Blindness and visual impairment remain serious public health problems. In order to further popularize and promote the ability of diagnosis, treatment, and prevention of hereditary eye diseases in China, professor Yin zhengqin from the Southwest hospital of the Army Medical University, professor Zhang qingjiong from Zhongshan Eye Center and other ophthalmologists and genetic scientists in China established the Chinese Arm of GEGC.

Grand occasion: On June 28, 2018, "The Founding Conference the Chinese Arm of GEGC" was grandly held in Chongqing, China. The conference was hosted by the CEGC and undertaken by the Southwest hospital of the Army Medical University in Chongqing. The purpose of the conference is to strengthen the communication and cooperation between China and other countries, further popularize and improve the ability of diagnosis and treatment of hereditary eye diseases in China.

At the beginning of the conference, a brief and grand opening ceremony was presided over by professor Qingjiong Zhang, the later chairman of the CEGC. The opening speech was delivered by president Zhengqin Yin, chairman of the CEGC. Then, Leilei Wang, a blind model, the representative of retinitis pigmentosa patients, gave a speech to describe her experience and thank CEGC with deep feeling. Takeshi Iwata, the current chairman of GEGC was invited to attend the opening ceremony and awarded the committee members formally. After the opening ceremony, the academic reports started. The experts from the USA, Japan, India, and other countries were invited to make a conference report:

Takeshi Iwata, President of GEGC, from the National Institute of Sensory Organs, Medical Center, National Hospital Organization Tokyo, Japan, made the speech of "Japan, Asia and the Global Eye Genetics Consortium: A research based consortium for advances in vision research."

Zhengqin Yin from Southwest Hospital, Army Medical University, China, talked about "Epidemiology study on the Bietti crystalline corneoretinal dystrophy in China."

Gyan Prakash, Previous President of GEGC, From National Eye Institute, National Institutes of Health Maryland, USA, emphasized the "Developing International Research Collaborations and New Program Areas of Interest including GEGC."

Sundaram Natarajan, General of GEGC, from Elect of All India Ophthalmology Society, Mumbai, India, had a report of "Updates on the genetics unit in Mumbai."

Zhenglin Yang, professor of Sichuan Provincial People's Hospital, China, informed of "The question and thoughts on Clinical genetic diagnosis of Ophthalmic genetics diseases."

Bo Lei from Henan Institute of Ophthalmology, China, overviewed "Progress in clinical trials of gene therapy for retinal diseases" et al.

These reports elaborate on the current hot issues of eye genetic diseases at home and abroad from different professional directions, which has aroused great interest and concern of the participants.

Then a brief closing ceremony was held In the afternoon, and the first committee meeting was held in the evening. Thus, The CEGC was formally established and the first eye genetic disease academic conference successfully concluded.

Council of the First Session

- *Zhengqin Yin*, PhD, President of Southwest Eye Hospital, Army Medical University, National "973" Chief Scientist. National Outstanding Youth Fund Winner.
- *Ningdong Li*, PhD, Professor of Beijing Children's Hospital (National Medical Center for Sick Children), the Affiliated Hospital of the Capital Medical University, Committee of the Chinese Ophthalmic Association, and ARVO.
- *Yang Li*, PhD, Director of Molecular Diagnostic Laboratory of Beijing Institute of Ophthalmology.
- *Zhenglin Yang*, PhD, Vice President of Sichuan Provincial People's Hospital, National Outstanding Youth Fund Winner.
- *Qingjiong Zhang*, PhD, Professor of Ophthalmology Center of Zhongshan University, National Outstanding Youth Fund Winner.
- *Zibing Jin*, PhD, Director of Genetic Eye Diseases Group of Affiliated Eye Hospital of Wenzhou Medical University, Director of Stem Cell Research Institute, Wenzhou Medical University.
- *Chen Zao*, PhD, Professor of Otolaryngological Hospital Affiliated to Fudan University of Shanghai, National Outstanding Youth Fund Winner.
- *Bo Lei*, PhD, Deputy Director of Henan institute of ophthalmology, Director of Henan ophthalmic disease clinical medical research center.
- *Ruifang Sui*, PhD, Professor of Peking Union College Hospital, Header of Eye Hereditary Disease and Visual Physiology Group.

Commissioners of the First Session

Zhuoshi Wang (PhD, Director of liaoning eye stem cell clinical application research center, Director of clinical research department, he shi ophthalmic hospital, shenyang), *Hui Wang* (Leader of Yunnan second people's hospital ophthalmology fundus disease group), *Panfeng Wang* (PhD, Professor of Zhongshan Ophthalmic Center of Sun Yat-sen University), *Xuyang Liu* (PhD, Professor of ophthalmology, Director of the ophthalmology laboratory, Chengdu west China hospital), *Xianjun Zhu* (PhD, Deputy director of the institute of experimental animals, Sichuan provincial people's hospital), *Zhengqin Yin* (PhD, President of Southwest Eye Hospital, Army Medical University), *Shen Yin* (PhD, Deputy director of the eye research institute of hubei provincial people's hospital), *Wensheng Li* (PhD, President of Shanghai aier eye hospital), *Ningdong Li* (PhD, Professor of Beijing Children's Hospital, the Affiliated Hospital of the Capital Medical University Committee of the Chinese Ophthalmic Association and ARVO), *Shiying Li* (PhD, Stem cell 2 group leader of Southwest Eye Hospital, leader of visual electrophysiology society of Chinese medical association), *Yang Li* (PhD, Professor of, Department of Eye Center of Beijing Tongren Hospital), *Lin Li* (PhD, Professor of Shandong provincial hospital of ophthalmology), *Bin Li* (PhD, professor of Department of ophthalmology, Tongji hospital of huazhong university of science and technology), *Rui Chen* (PhD, Director of the functional genomics center, Baylor College of Medicine), *Jianjun Chen* (PhD, Deputy director of ophthalmology of changzhou hospital of traditional Chinese medicine), Zhenglin Yang (PhD, Vice President of Sichuan Provincial People's Hospital), Liping Yang (PhD, Peking University Third Hospital), Qingjiong Zhang (PhD, Professor of Ophthalmology Center of Zhongshan University), *Zibing Jin* (PhD, Director of Genetic Eye Diseases Group of Affiliated Eye Hospital of Wenzhou Medical University Director, Stem Cell Research Institute, Wenzhou Medical Universit), *Chen Zhao* (PhD, Professor of Otolaryngological Hospital Affiliated to Fudan University of Shangha), *Shengping Hou* (PhD, Professor of the first affiliated hospital of chongqing medical university), *Huiping Yuan* (PhD, Professor of the second affiliated hospital of Harbin medical university) *Yonggang Yao* (PhD, Director of kunming institute of zoology, China), *Xun Sheng* (PhD, Professor of Ningxia eye hospital, people's hospital of ningxia hui autonomous region), *Zhipe Peng* (PhD, Professor of Shantou international eye center), *Bo Lei* (PhD, Deputy director of henan institute of oph-

thalmology, Director of henan ophthalmic disease clinical medical research center), *Ruifang Sui* (PhD, Professor of Peking Union College Hospital. Header of Eye Hereditary Disease and Visual Physiology Group).

The Annual Coordinators for 2018–2022

2018 *Zhengqin Yin* (PhD, President of Southwest Eye Hospital, Army Medical University).

2019 *Qingjiong Zhang* (PhD, Professor of Ophthalmology Center of Zhongshan University), *Yang Li* (PhD, Professor of, Department of Eye Center of Beijing Tongren Hospital).

2020 *Ruifang Sui* (PhD, Professor of Peking Union College Hospital, Header of Eye Hereditary Disease and Visual Physiology Group), *Zibing Jin* (PhD, Director of Genetic Eye Diseases Group of Affiliated Eye Hospital of Wenzhou Medical University, Director of Stem Cell Research Institute, Wenzhou Medical University).

2021 *Bo Lei* (PhD, Deputy Director of Henan institute of ophthalmology, Director of Henan ophthalmic disease clinical medical research center), *Ningdong Li* (PhD, Professor of Beijing Children's Hospital, the Affiliated Hospital of the Capital Medical University).

2022 *Zhenglin Yang* (PhD, President of Southwest Eye Hospital, Army Medical University), *ChenZhao* (PhD, Professor of Otolaryngological Hospital Affiliated to the Fudan University of Shanghai).

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4 Human Material for Research: Eye Banking, Biobanking and Ethical Access

Graeme A. Pollock, Christopher Hodge, Gerard Sutton, and Heather M. Machin

Abstract

In this chapter, we will explore access to human tissue for research and the role of the eye bank and biobank custodians responsible for this provision. We will examine the donation and allocation processes, discuss existing systems, donation and allocation pathways, the barriers to research allocation, and the ethical and the legal measures required to ensure that respect for the donation and the individual is maintained when providing tissue for research. Finally, we provide recommendations for researchers seeking human tissue for research.

Keywords

Eye banking · Biobanking · Ocular-research-tissue · Human tissue

4.1 Introduction

The use of ocular tissue for research purposes extends back several hundred years albeit the early means of acquiring tissue often lacked subtlety and ethical foundation [1]. An increasing requirement for human tissue for research projects has led to eye bank provision of ocular tissue for research, and in recent years, by biobanks. These services provide a platform for researchers and act as a direct link between donors, clinicians and researchers, thereby providing the efficient and ethical management of the ocular tissue, its storage and use in vision science [2].

G. A. Pollock · H. M. Machin (✉)
Lions Eye Donation Service, Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, University of Melbourne, Parkville, VIC, Australia
e-mail: graemeap@unimelb.edu.au; heather.machin@unimelb.edu.au

C. Hodge
New South Wales (NSW) Tissue Bank, NSW Organ and Tissue Donation Service, Sydney, NSW, Australia

Save Sight Institute, Discipline of Ophthalmology, Sydney Medical School, University of Sydney, Sydney, NSW, Australia

Graduate School of Health, University of Technology Sydney, Ultimo, NSW, Australia
e-mail: christopher.hodge@health.nsw.gov.au

G. Sutton
New South Wales (NSW) Tissue Bank, NSW Organ and Tissue Donation Service, Sydney, NSW, Australia

Save Sight Institute, Discipline of Ophthalmology, Sydney Medical School, University of Sydney, Sydney, NSW, Australia
e-mail: gerard.sutton@vei.com.au

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4.2 Demand for Ocular Tissue in Research

There are two fundamental uses for human ocular tissue; laboratory studies of anatomy, physiology, biochemistry, pathology, genomics and proteomics and the assessment of safety and efficacy in novel ophthalmic devices [3]. In vitro and animal studies will continue to play a role in the development of our understanding of ocular disease and treatment in general, however the unique anatomy of the human eye requires the direct assessment of human tissue to increase our understanding of particular disease processes such as glaucoma and macular degeneration [3–5]. Allocation of human eye tissue for research will be sourced from either existing Eye Banks or through Ocular Biobanks.

4.3 About Eye and Biobanking

The foundations of eye banking were laid by Filatov in 1937 with the recognition that donor tissue for corneal transplants could be recovered postmortem [6]. The first formal organised eye bank was perhaps the Eye Bank for Sight Restoration in New York, founded in 1944 [6]. At this time eye banks functioned as collection centres and simple storage facilities, holding whole globes in moist pot storage prior to corneal transplantation, which was usually undertaken within 24 h of donor eye retrieval. Today, eye bank responsibilities extend to the identification and medical assessment of deceased donors, consent to donation, surgical recovery, preservation and evaluation of donor tissue and the allocation and distribution of eye tissue. Primarily, eye banks are focused on the provision of tissue for transplantation, but also provide tissue for research, training and education purposes [7].

The provision of research tissue is a central tenet of the culture and service of eye banking and biobanking professions [8]. This practice may be traced to the origins of organised eye banking itself when many new eye banks were created as small service units of University departments. Thus, there is a long history of eye banks supporting vision research through the provision of donated human eye tissue for the purposes of research.

In comparison to eye banks, biobanks collect and store, for a prolonged duration, both living and deceased human biological samples for medical-scientific research purposes only. They are usually linked to phenotypic data [9]. Since their development through the early 1990s, biobanking has become essential to the work of precision medicine and generating advances in disease diagnosis and treatment [10]. More specifically to eye research, the collection of epidemiological data alongside ocular tissue in biobanks has led to a greater understanding of ocular pathology and the interplay between the eye and systemic disease.

4.4 The Eye Bank

Over the past few decades, eye banking systems have changed markedly and become increasingly complex and demanding, as have research approaches, programs and scientific techniques. These developments have conspired to increase the difficulty in providing research tissue that matches a researchers' requirements regarding tissue quality, type and clinical documentation [3]. An understanding of eye banks' systems and issues, and researchers' requirements alike, are required to create shared agreements on relevant practices and expectations [8].

4.4.1 Factors Affecting the Availability of Research Tissue

Donation and retrieval protocols, developed to assist tissue for transplantation need, alongside tissue for research needs, are not always well suited to meet the specific needs of researchers in terms of quantity, freshness, donor characteristics and preparation methods and availability [3]. In

turn, each of these has implications regarding the costs of providing the tissue [3].[1] Innovative collaborations with eye banks are required to address these issues [11, 12].

The donation and availability of deceased human tissue for research need to be viewed as a process rather than as an event. There are four steps during the process that affect the availability and type of tissue that becomes available for research, (1) Referral/Identification, (2) Consent and ethics, (3) Surgery of donation (Retrieval), and (4) processing practices.

1. *Referral*

   Eye donation referral systems integrate with the prevailing health system and thus vary across eye banks, jurisdictions and nations. Similarly, location will impact the potential demographic of the donor pool and the selection of donors, as does the location of the eye bank. Referral systems that focus on medical examiner/coronial cases or emergency rooms, generally manage younger donors with fewer chronic comorbidities and acute causes of death, and less clinical information is available. Large scale automated referral systems operate in some jurisdiction and are often shared across organ, tissue and eye donation services, which understandably focus on the primary mission of donation for transplantation. While less amenable and capable of the targeting of donors towards specific research requirements or indeed donors that would only be suitable for research purposes. This can be off-set by such systems generating or having the potential for, larger numbers of donors.

   The increasing complexity of referral systems and the number of donor organisations involved, and multi-tissue/multiorgan donations, invariably leads to issues around longer death-to-referral-to-retrieval times. Additionally, medical examiner/coronial cases are also subject to increasing regulatory and legal provisions with times for the release of the body for donation increasing. Indeed, it is known that some coronial jurisdictions may place at least a 24-hour embargo on donation.

2. *Consent*

   Depending on the jurisdiction, specific consent for research purposes may be required, in addition to consent for transplantation purposes. Furthermore, there is increasing ethical concern about the type and amount of information that needs to be conveyed or provided to the person providing consent for donation. More recently this particularly relates to the information provided regarding the potential uses of the donated tissue, including if the tissue is to be directed towards commercial uses or outcomes [7, 13].

   Both these issues mean that the person or institution directly responsible for consent has a direct influence on the amount of tissue available for research. The less that an eye bank is in control of this process (for example, in multiorgan or multi-tissue donations, it may be the Organ Procurement Organisation or Tissue Bank that is responsible for consent) increases the difficulty in ensuring that consent for research purposes is sought, and that the necessary information required to meet both legal and ethical concerns is appropriately provided.

   Eye banks require the researcher to provide evidence of ethical approval for their use of human tissue from their local human research ethics committee. When making submissions to the ethics committee it is then useful to work with the eye bank to ensure all ethical concerns regarding both donation and use have been addressed. Ensuring the number of tissues required for the research project can be adequately sourced is an essential prerequisite to the research planning stage.

[1] As services, regulations and access levels vary around the world. With many countries without access to eye bank or biobank services, we advise consultation with local, national or regional eye bank or biobank service provider or professional associations for allocation instructions specific to individual research needs.

3. *Surgery of Donation (Retrieval)*

   Not all eye banks will routinely operate on a 24-hour, seven-days-a-week basis, especially for the actual retrieval of donor tissue. This will directly affect the availability of tissue with short death to retrieval times. If the researcher requires shorter death-to-receival-times, they may have to negotiate special out-of-hours arrangements with the eye bank. During busy periods eye banks will prioritise retrieval of corneas for transplant purposes, so this can also lead to delays in the procurement of research tissue. Additionally, many eye banks now routinely perform in situ excisions of the cornea rather than whole globe enucleation. The Eye Bank Association of America reported that in situ excision was performed in 98% of all donations in the United States during 2016, restricting to less than 2% of all donations any other type of ocular tissue (e.g. retina, lens) being available for research [14]. Again, special arrangements may have to be negotiated with the eye bank, which like short retrieval times, will have additional considerations for both staffing arrangements and issues of consent.
4. *Processing of Eyes and Eye Tissue for Research*

   Within the eye banks, most tissue will be prepared initially for transplantation prior to becoming available for research. Eye banks may preserve their corneas in a hypothermic preservation media (short-term storage), or at normothermic temperatures using the organ culture technique (intermediate-term storage) [15]. If corneas are being provided directly from either of these preservation methods the researcher needs to consider what effect the death-to-preservation time, the solutions, the temperature and the time in storage may have on their outcomes.

   If a whole eye or a posterior pole is directly available for research purposes, it is usually a simple matter for the eye bank to prepare and place the tissue in a fixative or refrigerate state, and to make it available to researchers or place it with an ocular biobank.

   To maximise both the availability and the quality of the tissue, the researcher may have to make themselves readily available to prepare their research tissue. Alternatively, arrangements may be made for the eye bank staff to directly prepare the research tissue, but the researcher needs to consider issues of training, staff availability and priority, reagent quality control and cost. Researchers also need to consider transportation time, if the eye bank is distant from the research facilities.

4.4.2 Quality of Tissue

The quality and impact of scientific research is dependent on the quality of the tissue specimens available [2]. Quality in this instance usually relates to the time elapsed from the death of the donor until the tissue is prepared in a manner that is suitable for its research purpose. Although experiments involving histology, proteomics, cell isolations and culture for example, all have different degrees of tolerance for ischaemic times, all benefit from tissues in which death to preparation time is minimised. Increasingly this is an issue due to the high sensitivity of current assays, that Stamer and colleagues refer to as high-resolution 'omic' analyses (genomic, proteomic and metabolic) [5]. The most stringent requirements apply to mRNA preservation for gene expression studies [16].

A survey of members of the Association for Research in Vision and Ophthalmology (ARVO) published in 2018 indicated difficulty in obtaining eye bank tissue within a preferred death-to-preservation time (typically 6 h) [8]. Given the complexity of current-day eye donation and eye banking, that situation is unlikely to change. Therefore, in the planning phase, a genomic researcher needs to carefully consider their requirements regarding the assays to be performed [10] and the likely availability of tissue from different sources (including tissue from transplant procedure discard) [17–21].

4.4.3 Quality of Clinical Documentation

The medical history obtained by eye banks typically does not contain a full detailed ophthalmic medical history. The reasons are twofold; (1) The

ophthalmic history sought at the time of donation is only that which may affect the suitability of the cornea for transplantation, and (2) medical history available at the site of donation (e.g. acute care hospital, forensic pathology institute) usually contains no ophthalmic history at all. Eye banks must often rely on the donor's next-of-kin to provide any clues as to any ophthalmic history. Follow-up of such history can involve a good deal of detective work and involve searching for multiple histories that incorporate extended periods across the donor's lifetime. This is further complicated by issues of confidentiality, privacy and access to health records.

These issues need to be addressed at the time of consent for donation, and depending on the jurisdiction, the extent or validity of that consent to provide access to medical records that are otherwise 'out-of-bounds' in the determination of suitability for transplantation. It should also be appreciated that while the growing implementation of electronic medical records provides for easier and more efficient access to relevant clinical information, it still involves a considerable commitment of resources to provide the 'detective-work' involved. Often, for research purposes, this information will simply not be available.

4.4.4 Cost

Although freely given, donor eye tissues, whether it be for transplantation or research, are not identified, consented, retrieved, tested, processed and distributed for free. To ensure the sustainability of their services, eye banks need to be fiscally responsible and rely on service or processing fees to recoup not only their considerable operational costs but also their infrastructure and development costs. In this regard, researchers are benefitted from all the equipment, supplies and donor coordination activities that have been established for transplant donations, and thus the service fees that may be charged for research tissue are generally less than those charged for transplant tissue. Nevertheless, depending on the researcher's requirements, the additional resources and procedural changes involved in providing research tissue can be quite considerable. These may involve changes to identification and referral processes, additional requirements for consent, provision of a 24-hour retrieval service and associated out-of-hours costs, an additional collection of ophthalmic medical records, preparation of research tissue by the eye bank, and delivery costs.

Of consideration, the typical cost of obtaining a whole globe for research in US dollars, averaged $481 ± $572 (range, $0–$3000) [3, 11]. Importantly, researchers now need to budget properly for tissue costs in grant applications and work directly with the eye bank during this process.

4.5 The Biobank

4.5.1 Accessing Research Tissue (Models of Biobanks)

With an increase in research and technology, biobanks represent an excellent opportunity to develop key research initiatives with numerous examples within ophthalmology ranging from small, specialised centres to large data and tissue-driven projects. Specialists or interest groups involved in the treatment of rare ocular diseases such as uveitis or ocular cancers will often drive sub-specialty repositories. These biobanks are small and regularly situated within affiliated teaching hospitals or universities providing storage and access. Given direct links to specialists, specimens are often accompanied by significant clinical and treatment data representing a valuable resource. The sharing and scalability capabilities of these biobanks are often limited, however. With access to tissue and established working relationships with both surgeons and researchers, eye banks often provide biobank opportunities. The advantage of an eye bank presence within biobanking is the provision of standard Good Manufacturing Practices and reliable and robust protocols providing an excellent foundation [22].

As genetic or tissue sampling may not be sufficient to identify biological processes, the corresponding use of big data alongside biobanks represents a key initiative in developing our under-

standing of ocular disease [23]. Outside the obvious advantages of information access, these large collections bring significant financial and ethical considerations. Established large scale ocular repositories such as the UK Biobank must rely on significant funding from external non-government sources and grant opportunities to continue. As a primary source of information for local and external researchers, strict criteria for the use of data and tissue is essential to avoid a range of potential ethical concerns including consent, de-identification and both tissue re-use and disposal.

Of note, access to tissue is usually determined across most Biobanks by a central research or management committee which requires researchers to meet specific criteria for scientific quality and public interest [23]. Similar to the access of eye tissue through eye banks, formal human ethics approval is considered a pre-requisite for access to tissue.

Biobanks, by reducing the time and funds needed by individual researchers to collect, store and curate samples, help facilitate access to tissue and thereby the timely translation of research findings into improved patient outcomes [24].

4.5.2 The Consent Process (Living and Cadaveric)

The process and information will be dependent on the status of the donor. The traditional model of informed consent, imposed by the Declaration of Helsinki and the Council of Europe Oviedo Convention 1997, provides a significant framework for both researchers and biobanks, however, there remain complex ethical, legal and funding challenges which require ongoing consideration [25].

The living donor offers biobanks an opportunity to acquire epidemiological information and access to pathology specimens following treatment [26]. In the context of broader epidemiological research, such as the within the UK Biobank, the donor may be viewed more appropriately as a voluntary participant providing information about general and ocular health to supplement clinical investigations including ophthalmic assessment and or the collection of tissue samples such as blood, saliva or tears for additional genomic or systemic analyses. Drawn mostly from general, healthy populations, participants in epidemiological research will be aware of the commitment to the required project albeit providing adequate information remains a necessity prior to consent. The acquisition of residual ocular pathology following treatment represents a significant opportunity for researchers, especially with adjunctive clinical and patient history available. Although there has been minimal research investigating patient willingness to donate residual ocular tissue, research in other areas suggests most participants will readily support the collection of biospecimens regardless of the tests required or the scope of consent [27, 28]. Of consideration, Critchley and co-authors in a cohort of cancer patients found that although most patients viewed the actual consent process as important, this was secondary to being provided an understanding of the ongoing donor confidentiality, the study's ethical oversight and overall contribution to healthcare [29]. The possibility of tissue sharing with other researchers or international use, obtaining feedback from genetic tests and public education were additional concerns and represent a fundamental obligation for inclusion within the patient information and consent process [30].

The pathway for the acquisition of ocular tissue for research purposes from deceased donors is relatively well defined albeit with additional ethical considerations. Eye banks continue to play a prominent role in the acquisition of ocular tissue for research purposes. Although ocular tissue for transplantation remains a priority, prior counselling within the clinic, especially for patients with rare ocular conditions may provide a direct pathway to increase biobank material. Indeed, a proposed eye donation registry for research, in the form of an advanced directive has previously been considered and has been well received by ophthalmic patients, their family members and eye care providers [11, 31]. In a survey of eye care practitioners, Williams et al.

found that the majority (62%) would feel comfortable discussing this option with patients [11]. University-affiliated programs such as through Duke University in the USA, currently provide an option for an 'anatomical gift act' which facilitates the donation of deceased tissue for research purposes [1]. In this way, the consent process largely mirrors that of the living participant.

Donor status, living or deceased, will govern the consent process for biobank tissue. At minimum verbal consent to research must be recorded through the donor conversation.

4.5.3 Legislation and Ethics

Whilst ocular biobanks will differ in size, the risks associated with the collection of tissue and personal information remain consistent. The ethical concerns encountered often represent a mixture of technical and quality issues and of societal dimensions [7, 32]. These include the protection of the rights of the donors' autonomy and confidentiality following sample acquisition, the risk of unexpected consequences of research, the future use of the samples and ensuring the non-commercial use of ocular tissue and the maximum quality of sample conservation [32].

The conversation of tissue ownership following donation represents a potential moral dilemma for biobanks. Respect for the donor remains paramount and this will be supported through specific consent information regarding the process of tissue use and an ongoing dialogue between researchers and the public to extend trust in scientific research [33].

There remains a risk that the use of new techniques, particularly the exploration of genetic information, will continue to outpace currently available protections and lead to an increased risk of donor identification or the discovery of incidental findings that may potentially impact the donor or next of kin (NOK) [34–36]. Prior discussion of ongoing anonymity and acknowledgement of the donor or NOK to receive feedback is essential.

The practical difficulty of using samples after a lengthy time lapse is significant, representing a distinction from eye banks [37]. With rapidly developing technology and increasing collaboration, it remains almost impossible to provide a donor or NOK with all avenues for future sample use. Although re-consent with the living donor or NOK in deceased situations may represent an appropriate option ethically, this leads to further considerations. Practically this is difficult and may represent a significant financial and logistical burden upon either researchers or the biobank directly [38]. Contacting the NOK may cause emotional distress over time. Mandating the provision of counselling for relatives in this situation has been discussed by ethics committees previously. The obvious solution is to provide a broad consent, and this is supported by the World Health Organisation which considers this option as the most efficient and economical approach [13]. Opponents to this approach suggest the process undermines the meaning of consent which by nature requires precise information [39]. This represents an ongoing debate with a more targeted approach based on the type of donation the probable option [33].

Research has increasingly been moving towards globalisation. A relevant example is the availability of external research collaboration across the UK Ocular Biobank to share samples and data from donors. This suggests it is imperative that a legal framework is provided by individual biobanks at inception, not only for sample access by international researchers but also for the exchange of biological material between countries [40, 41].

A number of barriers exist to limit the potential scope and services provided by ocular biobanks requiring consideration from both the researchers and biobank.

4.5.4 Barriers to Biobanking

Ethical and legislative concerns aside, perhaps the greatest barrier for researchers gaining access to human tissue remains that the major route of access to tissue is predicated on the altruism of the donor to provide this generous

gift. As discussed previously within the chapter, the process of organ donation represents a highly emotive and difficult conversation for most families with additional factors such as culture and religious beliefs playing a further role in the decision to donate [33, 42]. Maintaining a focus upon providing tissue for transplantation is essential for eye banks therefore the available secondary pool of tissue for research and the required supplementary patient information will be minimal at best [5]. Along similar lines, although ocular disease will not immediately exclude patients from donation, coordinators will invariably screen out many patients with concurrent ocular pathology. This can lead to a relative homogenisation of available tissue limiting researchers from developing an understanding of the ocular disease and its progression [23, 43]. A similar issue is seen within large biobanks that enroll from a general, mostly healthy population.

Outside of the semi-structured NOK conversation for deceased donors, the opportunity to participate in biobank research or donation is seldom discussed [24]. Involving the clinician represents a potentially significant option, especially in rare ophthalmic conditions however this may be beyond the capacity, or interest of a busy private clinician, particularly without research affiliations or interest. Biobanks must therefore continue to establish working relationships across ophthalmology to optimise the potential reach of the projects.

As stated in the WHO Guiding Principles on Cell, Tissue and Organ Transplantation, 'All health care facilities and professionals involved in tissue procurement and transplantation procedures should be prohibited from receiving any payment that exceeds the justifiable fee for the services rendered' [13]. Most biobanks receive financial support from state research programs through start-up in the early years, with a subsequent expectation of future self-sufficiency. Cost recovery through the initial retrieval and storage process is justified and relatively well managed however as samples deposited in biobanks are stored for research for a much longer time and may involve additional preparation, more complex funding models will be required [44, 45]. Another solution for biobanks could be to establish partnerships with private biomedical companies or non-government organisations to ensure ongoing financial support. This may however inflate the perceived risk of a reduction in biobank autonomy and propose further ethical and legal concerns to the community and donor. Providing transparency between biobanks, researchers and the community is therefore essential. Amalgamation of several smaller biobanks offers additional opportunities to manage financial constraints however this may still not be enough, as evidenced by the closure in 2016 of the centralised donor tissue program Foundation Fighting Blindness [5].

4.6 Recommendations

Researchers seeking human tissue are advised to follow several guidelines when planning and working with both eye banks [3] and biobanks:

- *Availability*: researchers should become acquainted with the limitations of an eye bank's demographics and processes (donor pool demographics, ophthalmic conditions, death-to-preservation times, in situ versus enucleation retrieval, preservation system, extent of clinical documentation).
- *Costs*: researchers should budget properly for tissue costs in grant applications.
- *Death-to-Preservation Times*: researchers should be practical and flexible, especially if fresh or rare tissues are required. Be prepared for contact on weekends or odd hours.
  - In the planning phase, a genomic researcher needs to carefully consider their requirements regarding the assays to be performed which will inform death-to-preservation times required.
- *Ethics Approval*: researchers should also ensure that the procurement and use of donated human ocular tissue meets the bioethical standards required by the wider ophthalmic and tissue donation community.
- *Preparation*: The ability of an eye bank to meet specific needs is protocol-dependent and

should be discussed in advance. Be clear about experimental needs and expectations.

4.7 Summary

Both eye banks and ocular biobanks provide researchers with an opportunity to accelerate important clinical discoveries leading to potential diagnostic and treatment benefits for patients. Understanding the complex ethical and legal considerations supporting the collection of information and tissue is essential to maintaining our obligations to the donors who provide this invaluable resource.

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5 Current Understanding of Polypoidal Choroidal Vasculopathy

An Overview on Clinical and Genetic Aspect of Polypoidal Choroidal Vasculopathy

Sandeep Kumar and Yingbin Fu

Abstract

Polypoidal choroidal vasculopathy (PCV) is a common subtype of age-related macular degeneration (AMD) in Asians. PCV is characterized by branching vascular networks and polypoidal lesions in the choroidal vasculature. Since it was first described four decades ago, there is significant progress in the diagnosis, etiopathogenesis, and treatment of PCV. The progress was driven by the advancement of multimodal imaging including indocyanine green angiography and optical coherence tomography, genome-wide association studies, and animal model investigations. There is clear evidence that PCV has distinct clinical characteristics, natural histories, and treatment outcomes compared with the wet type AMD that is typical in Western populations. In this review, we summarize the current understanding of PCV with a focus on the parallel studies from the clinical setting and animal models.

Keywords

Polypoidal choroidal vasculopathy · Age-related macular degeneration · Choroidal neovascularization · Exudative age-related macular degeneration · Pachychoroid neovasculopathy

5.1 Introduction

Age-related macular degeneration (AMD) is a leading cause of irreversible blindness in the elderly. Approximately 8.7% of the worldwide population suffers from AMD, with the number of cases expected to rise from around 196 million in 2010 to around 288 million in 2040 [1]. Wet AMD (also called neovascular AMD, or nAMD) includes choroidal neovascularization (CNV) and polypoidal choroidal vasculopathy (PCV). CNV describes the growth of new blood vessels from the choroid into the subretinal space whereas PCV refers to choroidal vessel abnormalities (e.g., polypoidal dilations) [2]. PCV is a common subtype of wet AMD in Asian populations while CNV is the typical subtype in Western populations. PCV is frequently associated with recurrent hemorrhagic or exudative pigment epithelium detachment (PED). The clinical course of PCV

S. Kumar
Ophthalmology Department, Absorption Systems, San Diego, CA, USA

Y. Fu (✉)
Department of Ophthalmology, Baylor College of Medicine, Houston, TX, USA
e-mail: yingbin.Fu@bcm.edu

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is generally more stable and visual outcomes are more favorable compared with CNV. The current anti-VEGF therapy is less effective in treating PCV compared with classic CNV. Research from genetic, clinical, and animal model investigations have shed light on the pathophysiological mechanism of PCV. We will discuss these areas in this review.

5.2 Epidemiology and Global Perspective

PCV has ~four-fold higher prevalence in pigmented races (e.g., African, Japanese, Chinese, and other Asians) than the non-pigmented races like Caucasian [2]. This is clearly opposite to the incidences of CNV in the Caucasian population. The estimated prevalence of PCV is 22.3%–61.6% among Asians [3] in contrast to 8%–13% of CNV in Caucasians [4]. There is a marked male preponderance of 63%–78.5% and only 5.9%–24.1% have bilateral disease. In Caucasians, women are predominantly affected at a ratio of 4.7:1 [4]. PCV is generally diagnosed in patients between the ages of 50 and 65 years though it can range from the 20s to 80s. The average affected age among the Korean, Chinese, Japanese, and the Indian population is 60–65 [5]. The average age of onset in Caucasians is 75.4 years [6]. 92% of Japanese patients' PCV occurs in the central macula, whereas there is an even distribution of macular and peripapillary location in Europeans. Only 14% of Japanese patients develop bilateral disease, in contrast to 32% of Europeans [2].

5.3 Etiopathogenesis

Smoking is a known risk factor for AMD and also appears to be an important risk factor for PCV. Two population-based studies in the Japanese demonstrated that cigarette smoking is associated with an odds ratio of 4.4 and 4.87 for PCV when compared with normal controls [7, 8]. Various inflammatory cytokines and systemic factors are associated with PCV and CNV and may cause PCV and CNV by compromising the capacity of the immune system to handle immunological stress and resulted in an immune imbalance. Systemic serum biomarker analysis has been used to differentiate between PCV and CNV. Subhi et al. found that inflammatory C-reactive protein (CRP) protein in the plasma of PCV patients was elevated but other inflammatory cytokines interleukin (IL)-1β, IL-6, IL-8, IL-10, and tumor necrosis factor receptor 2 (TNF-R2) were similar to the healthy controls [9]. Other studies reported elevated proinflammatory cytokine levels, including IL-1b and IL-23, in aqueous and vitreous samples, which support a role for inflammation in PCV [10, 11]. Increased plasma homocysteine levels are linked to retinal diseases such as retinal vascular occlusion and diabetic retinopathy [12–14]. In the Chinese PCV population, 1 μmol/L of increase of plasma homocysteine to the basal level increases the 1.5-fold risk to develop PCV [15]. Because higher levels of homocysteine had been linked to endothelial injury and increased oxidative stress [16, 17], it was hypothesized that elevated levels of homocysteine may induce injury to choroidal arteries and cause aneurysmal like dilations (polyps) and arteriosclerosis of choroidal vessels in PCV [15]. In addition, increased levels of matrix metalloproteinase (MMP 2 and MMP 9) were detected in PCV lesions, and both MMPs were increased in the serum of PCV patients, suggesting that they may have a role in the pathogenesis of PCV [18, 19].

In Clinic, PCV patients typically presented with inner choroidal vasculature abnormalities accompanied by extensive exudation, bleeding, and proteinaceous leakage followed with lipid deposition from active polypoidal vascular lesions [2, 20]. Surgically extracted specimens from PCV patients showed thickened and complete or partial obstruction of hyalinized choroid vessels walls due to the extravasation of plasma protein and deposition of basement membrane-like material beneath the Bruch's membrane [21, 22]. Stagnation of blood was evidenced by the presence of blood cells in the vascular cavity, and adherence of neutrophils to the inner ves-

sel walls [22]. Microscopic examination identified degenerative changes in the inner elastic layer and arteriosclerotic nature of the choroid vessels. An increase of deposition of basement membrane-like material together with collagen fibers in the arteriolar walls was also featured in the PCV specimen [23]. Complete or varying severe to a partial loss of α-SMA was detected in the hyalinized vessels in the PCV specimen [22]. Moreover, exudative changes around the vessels were more significant in PCV compare to the CNV portion of the excised specimen. CD34, an endothelial cell marker, immunostaining was found to be discontinuous in PCV while the CNV portion of tissue was presented with in continuity in vascular endothelium [22]. Recently based on the histopathological features of the autopsy tissue obtained from a 60-year-old African American woman with PCV, Tso M et al. suggested that PCV may be a venous stasis choroidopathy condition [24]. They observed that PCV is composed of dilated vascular channels consisting of thin wall venules intertwined with arteriosclerotic choroidal arterioles. Occlusion of these choroidal vascular channels might give rise to choroidal stasis and ischemia leading to serous RPE detachment and a sub-RPE neovascular membrane. Gross dilatation of the choroidal venules and capillaries in the sub-RPE neovascular membrane leads to the characteristic "grape like" structures, a unique clinical feature in this disease entity. Tso M et al. hypothesized that choroidal venular stasis is one of the primary causes of PCV pathogenesis.

Based on genome-wide association studies implicating the involvement of high temperature requirement factor A1 (HTRA1), a multifunctional secreted serine protease that is ubiquitously expressed in mammalian tissues, in AMD including PCV [25–29], we generated the first PCV model by transgenically expressing human HTRA1 in mouse RPE [30, 31]. Increased expression of HTRA1 induced two key features of PCV, polypoidal dilations (polyps) and branching vascular network (BVN), in transgenic *hHTRA1*$^{+}$ mice. BVN (Fig. 5.1a, red circles) and polyps (Fig. 5.1a, blue arrows) begin to appear ~1 min after ICG injection in the early phase (0–4 min) and become more distinct in the middle phase (6–15 min) and late phase (18–22 min) with the fading of the choroidal vasculature. More lesions started to appear in the middle phase (Fig. 5.1a, black arrows). On funduscopy, polypoidal lesions appear as reddish-orange nodules (Fig. 5.1b, middle row, white arrowhead; bottom row, red box). A cluster of polypoidal lesions, which faded at the late phase of ICGA, appears on the fundus as a cluster of reddish-orange nodules (Fig. 5.1b, bottom row, red box). Hemorrhagic (Fig. 5.1b, middle row, white stars) and serous (Fig. 5.1b, bottom row, white asterisks) PEDs, RPE degeneration (Fig. 5.1b, bottom row, yellow arrow) as well as yellowish hard exudates (Fig. 5.1b, middle row, green arrow) were observed near the lesion site. These phenotypes share remarkable similarities to the well-established clinical features of human PCV. By performing comprehensive genetic, histopathological, imaging, and molecular biological studies on the *hHTRA1*$^{+}$ PCV mouse model in combination with analysis on human PCV specimens, we demonstrated that HTRA1 mediated degradation of elastin in choroidal vessels is critical for the development of PCV, which exhibited destructive extracellular matrix remodeling and vascular smooth muscle cell loss [18]. Compared with weak PCV, severe PCV exhibited prominent immune complex deposition, complement activation, and infiltration of inflammatory cells, suggesting inflammation plays a key role in PCV progression. Based on this study, we proposed a two-stage process for PCV pathogenesis: PCV initiation is mediated by increased HTRA1 activity while progression is driven by chronic inflammation.

5.4 Clinical Features

Although both PCV and CNV are related to choroidal vasculature, they are different in clinical nature. In CNV, abnormal choroid vessels break the Bruch's membrane (BM) and grow into the sub-RPE or subretinal space, while PCV arises

within the inner choroidal vasculature and characterized by the formation of branching vascular networks (BVN) that terminates in aneurism like polypoidal lesions. PCV was characterized as a variant of a type 1 neovascularization in which the abnormal choroid vessels are located in the sub-RPE space [2]. In the early phase of PCV, patients typically presented with extensive subretinal exudation and bleeding with minimal cystic changes and negligible impact on the retina function. PCV may progress to an advanced phase very quickly due to proteinaceous leakage followed with lipid deposition from active polypoidal vascular lesions with a significant impact on the retinal function [2, 20].

Although fluorescein angiography (FA) is routinely used in the diagnosis of CNV, the use of FA in PCV is limited since FA is not able to reli-

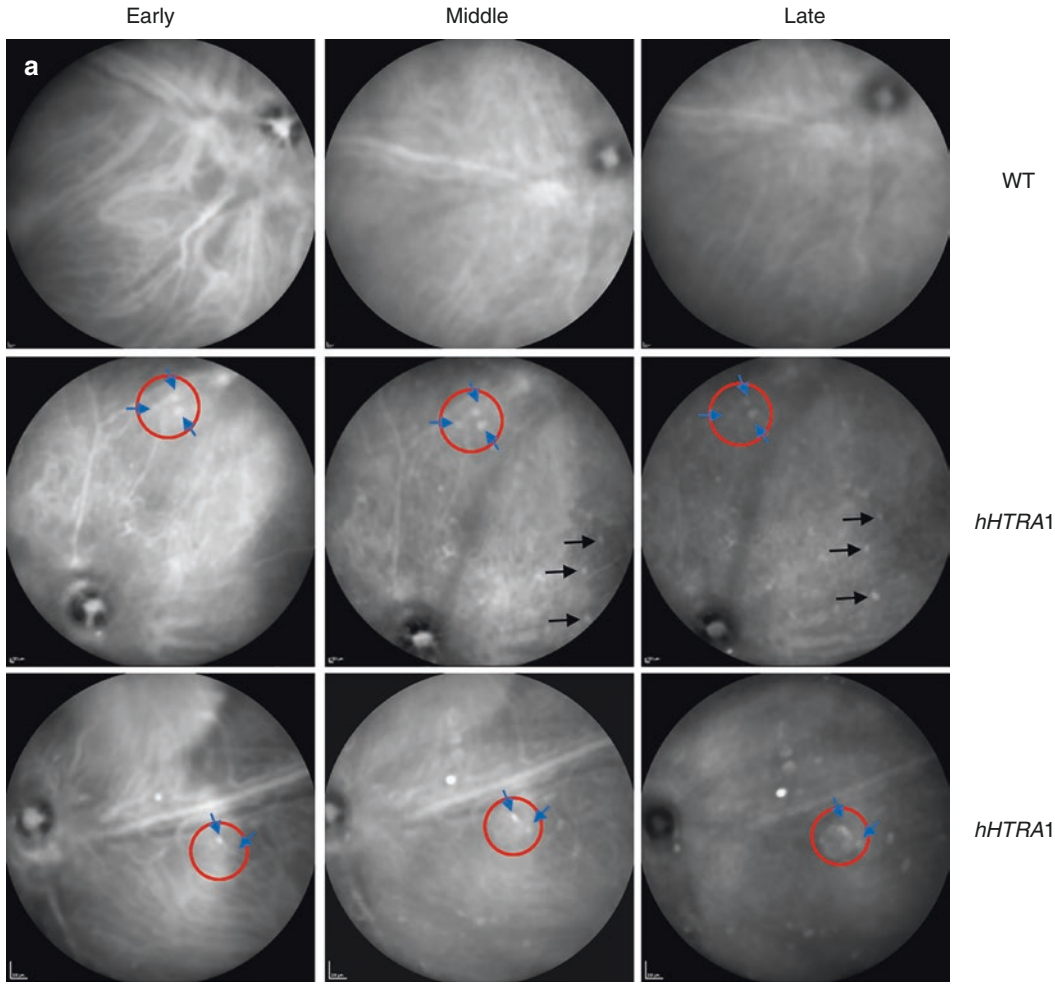

Fig. 5.1 ICGA and funduscopic features of PCV lesions in *hHTRA1*$^{+}$ mice. (**a**) Angiographic features of *hHTRA1*$^{+}$ mice on time course ICGA. The early, middle, and late phases of ICGA were recorded for wild-type (WT) control and *hHTRA1*$^{+}$ mice. *hHTRA1*$^{+}$ mice developed polyp dilations (blue arrows) and BVN (red circles) from the early phase. More lesions started to appear in the middle phase (black arrows). (**b**) Funduscopic examination of WT control and *hHTRA1*$^{+}$ mice. In *hHTRA1*$^{+}$ mice, reddish-orange nodules, which correspond to PCV lesion structures based on ICGA, are indicated (middle row, white arrowhead; bottom row, red box). Hemorrhagic (middle row, white stars) and serous (bottom row, white asterisks) PEDs, RPE degeneration (bottom row, yellow arrow) as well as yellowish hard exudates were observed near the lesion site (middle row, green arrow). Reproduced from Invest. Ophthalmol. Vis. Sci. 55, 3842–3850. Copyright the Association for Research in Vision and Ophthalmology

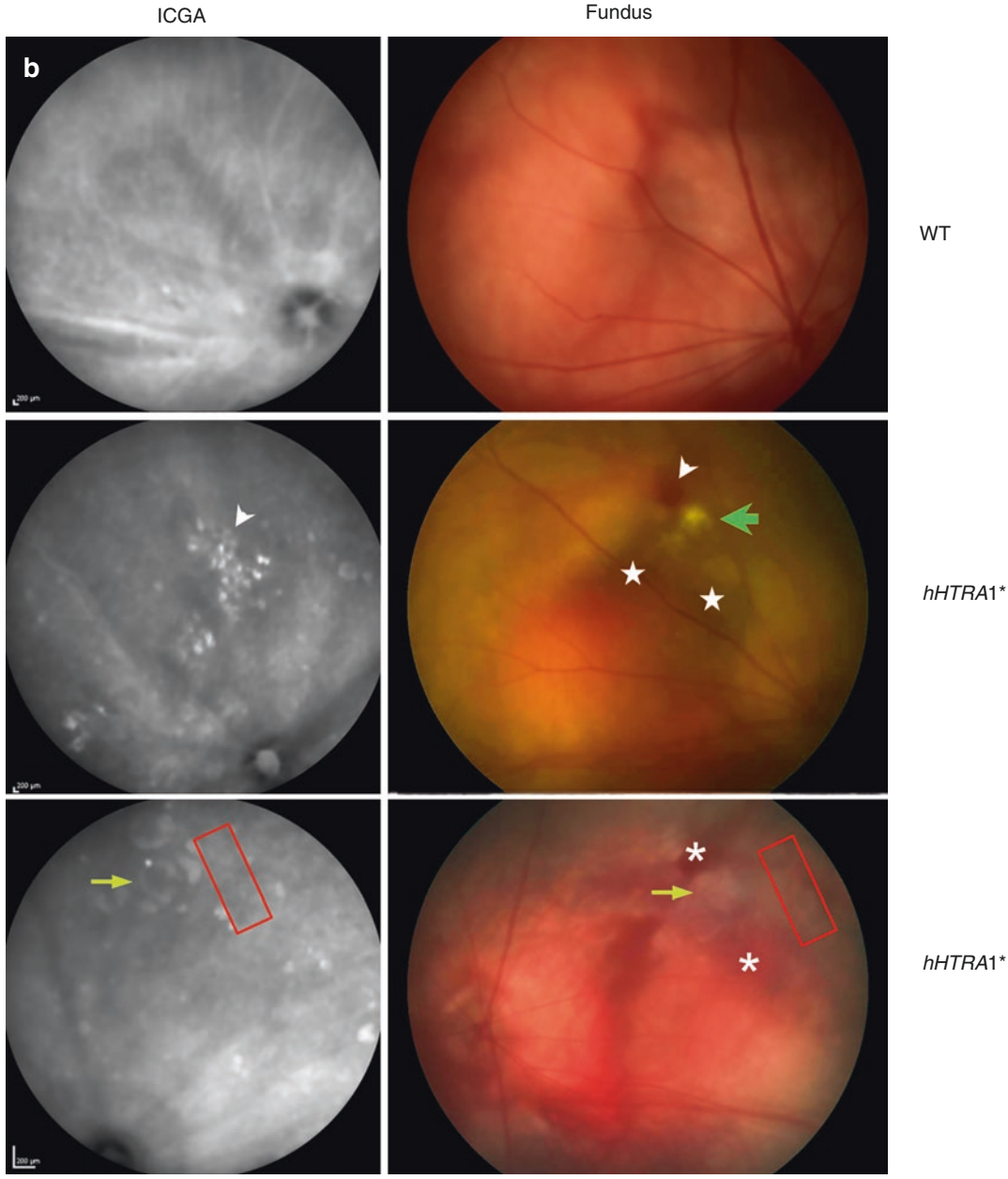

Fig. 5.1 (continued)

ably detect polys. Indocyanine green angiography (ICGA) is considered the gold standard for the diagnosis of PCV due to its capability to image the posterior choroidal vasculature [2]. Polyps (single or multiple) can be detected in the early phase of ICGA. BVN and other features can be visualized with confocal scanning ophthalmoscopy. On fundoscopy, the presence of orange-red subretinal nodules with corresponding ICG hyperfluorescence is characteristic of PCV [32]. Both FA and ICGA are required to differentiate PCV from CNV. FA can detect occult choroidal neovascularization (CNV) while ICGA can visualize of the abnormal polypoidal lesions.

Optical coherence tomography (OCT) imaging is especially useful to detect subretinal fluid, PED, and polypoidal lesions [2, 33]. In addition to BVN and polyps, other clinical features such as late geographic hyperfluorescence (LGH) [34, 35] and hyperfluorescent plaque [36, 37] have been visualized in PCV eyes based on ICGA.

By examining the PCV phenotypes (e.g., lesion type, distribution) of the PCV model *hHTRA1*$^{+}$ mice by a variety of in vivo imaging techniques (ICGA, funduscopy, and SD-OCT), we found the *hHTRA1*$^{+}$ mice exhibit additional features that are present in PCV and wet type AMD, e.g., LGH, plaque, and PED, in addition to BVN and polyps [31]. SD-OCT located the lesions in the choroid while round protrusions of the RPE can be detected, which is consistent with polypoidal lesions. It is intriguing that male *hHTRA1*$^{+}$ mice exhibit more severe types of lesions (e.g., LGH and PED) than females. This is reminiscent of the higher incidence of PCV in males than females in Asians although the opposite is true for Europeans [2, 3]. In particular, we are the first to perform ICGA on PCV animal models by tail-vein injection of ICG to obtain high-quality ICGA comparable to human studies in terms of the three phases (early, middle, and late) of angiography [31, 38]. By using this technique, the polyps can be detected in the early "fill-in" phase of ICGA, most lesions become visible in the middle phase and more distinct in the late phase with the fading of surrounding vessels (Fig. 5.1a). This technique is also useful to distinguish between different types of lesions, e.g., LGH vs. plaques. This animal model will provide an invaluable tool for future mechanistic and translational studies (e.g., drug screening) of PCV and other forms of choroidal vasculopathies.

Recently, a new clinical entity of type 1 neovascularization termed pachychoroid neovasculopathy associated with choroidal thickening, but lacking soft drusen and other typical AMD findings, was reported [39]. Some investigators suggest PCV falls within the pachychoroid spectrum of conditions including pachychoroid pigment epitheliopathy, central serous chorioretinopathy, and PCV [40–43]. These studies provide some evidence that PCV is a pachychoroid-driven disorder with findings of similar choroidal features and the occurrence of polypoidal lesions in eyes lacking typical AMD features [36, 42, 44]. However, several studies also suggested that pachychoroid as an underlying cause for focal choroidal excavation [40], geographic atrophy named as pachychoroid geographic atrophy [43], peripapillary exudative changes named as peripapillary retinoschisis [41], peripapillary pachychoroid syndrome [45], and pachydrusen [46]. Further studies are necessary to clarify the relationship between pachychoroid neovasculopathy and PCV.

5.5 Genetic Aspects

Genetic association studies from Chinese and Japanese populations indicated that genetic loci related to AMD such as the complement cascade, inflammatory pathway, extracellular matrix/basement membrane regulation pathway, and lipid metabolism are associated with PCV [3, 47]. A recent study on the SNP meta-analysis in East Asian population revealed that eight genes linked to CNV including *HTRA1*, age-related maculopathy susceptibility2 (ARMS2), complement system factor H (CFH), factor B (CFB), component 2 (C2), Super killer viralicidic activity 2-like (SKIV2L), and cholesterol ester transfer protein (CETP) are also significantly associated with PCV [48]. Particularly, numerous studies have shown that genetic loci in chromosome 10q26 surrounding HTRA1 and ARMS2 are strongly associated with AMD including PCV [25, 27–29, 49–52]. However, a series of studies on the influence of AMD-associated polymorphisms on the expression of ARMS2 and/or HTRA1 have yielded conflicting results [29, 53–58]. However, recent studies started to provide evidence that variants in the promoter region of HTRA1 can transcriptionally upregulate HTRA1 [59, 60]. Transgenic expression of HTRA1 or ARMS2 in mouse has shown that overexpression of HTRA1 but not ARMS2 induced PCV and CNV [30, 31, 59, 60]. Furthermore, we showed that HTRA1

protein was significantly increased in RPE and degenerating choroidal vessels of PCV lesions in human specimens, suggesting HTRA1 likely plays a causal role in PCV pathogenesis [18]. Interestingly, a rare missense (Lys329Arg) variant of the FGD6 gene in the Han Chinese population was found to be significantly associated with PCV but not with CNV. FGD6-Arg329 promoted more abnormal vessel development in the mouse retina than FGD6-Lys329, suggesting that oxidized phospholipids and FGD6-Arg329 might act synergistically to increase susceptibility to PCV [61]. A GG missense variant at rs5882 in the CETP locus was found to have a 3.53-fold increased risk of PCV compared with the AA genotype. PCV patients with the rs5882 GG genotype had lower serum high-density lipoprotein levels than the AA genotype [62]. The CFH Y402H polymorphism might also have a synergistic effect on cigarette smoking to further increase the risk of PCV [63]. The c.6196A > G variant in the IGFN1 gene was found to be significantly associated with only PCV (combined $p = 7.1 \times 10^{-11}$, odds ratio = 9.44), but not with CNV (combined $p = 0.683$, odds ratio = 1.30). The minor allele G conferred an increased risk of PCV [64].

5.6 Clinical

Depending on the state of PCV (active or inactive), several treatment options, e.g., thermal laser photocoagulation (TLP), verteporfin PDT (vPDT), anti-VEGF therapy, and various combinations of these therapies are available. ICGA-guided direct TLP, which targets both polyps and BVN (whole lesion with polyps), has been shown to either stabilize or improve the vision. However, Recurrence of polyps, the formation of subsequent CNV, exudation or hemorrhage, and atrophy at the fovea have been observed with TLP therapy [65–67]. In vPDT, verteporfin (a photosensitizing agent) produces a photochemical reaction when activated by nonthermal laser in the far-red spectrum and produces selective vascular occlusion by thrombosis [68]. Visual outcome of vPDT treated PCV eyes was stable for 2 years but the effect gets diminished with time and PCV re-occurs within 3–5 years post vPDT. Post-PDT subretinal hemorrhage, massive suprachoroidal hemorrhage, RPE tears, and microrips at the margin of the PED are the reported complications of PDT for PCV. PDT alone is ineffective in causing regression of the BVN or in resolving exudative activity arising from the BVN, but when combined with anti-VEGF compounds demonstrated better visual outcomes [69, 70]. Anti-VEGF drugs, bevacizumab (a full-length anti-VEGF antibody) or Ranibizumab (an antibody fragment with smaller size), decreases the exudation and improve or stabilize vision but has minimal to no change in polyp regression [71, 72]. The newer anti-VEGF drug, Aflibercept (a soluble decoy receptor fusion protein consisting of the binding domains of VEGF receptors 1 and 2), demonstrates improved visual outcome and causes poly regression [73–75]. However, long-term study is needed to fully assess the efficacy of this treatment. Pigment epithelial tears, post-injection subretinal hemorrhage and vitreous hemorrhage, and RPE atrophy are few complications reported. Because anti-VEGF drugs reduce the exudation from polypoidal lesions arising from the BVN and vPDT causes thrombosis of the polypoidal lesions, a combination of the two therapies produces better long-term visual outcomes. In the EVEREST study, PDT and ranibizumab combination increased the polyps closure rate to (77.8%) compared to PDT alone (71.4%) [76] whereas Ranibizumab monotherapy can close only 28.6% polyps. In addition, PDT alone is ineffective in causing regression of the BVN or in resolving exudative activity arising from the BVN [76]. The EVEREST II study revealed that the combination therapy (PDT with ranibizumab) achieved superior BCVA gain (8.3 vs. 5.1 letters; $p = 0.013$), along with superior anatomical outcome, including higher polyp closure rate (69.3% vs. 34.7%; $p < 0.01$) and a higher proportion with the absence of disease activity (79.5% vs. 50.0%) at month 12 compared with ranibizumab monotherapy [77].

5.7 Summary

Significant advances have been made in our understanding of PCV in terms of genetics, pathophysiology, and treatment strategy. We have gained improved knowledge regarding the difference between PCV and CNV. The principal therapies for PCV are laser photocoagulation, PDT, and anti-VEGF drugs. The best-reported treatment combines PDT with anti-VEGF drugs [78]. The combination therapy of PDT and anti-VEGF drugs have achieved good results in polyp closure. However, one major concern regarding PDT is the high rate of recurrence or the development of new polypoidal lesions [69, 70]. On the other hand, long-term use of anti-VEGF therapy can lead to anti-VEGF resistance [79–81], and long-term blockade of VEGF signaling in retinal diseases may have detrimental side effects [82, 83]. Therefore, the development of novel drugs that prevent or reduce both BVN and polypoidal lesions could have a considerable impact on the current therapeutic strategy. Animal models have played an essential role in the development of anti-VEGF drugs for CNV. The availability of a PCV animal model should facilitate the development of new treatment for PCV [18, 31].

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Genetics of Age-Related Macular Degeneration in Asia

6

Kenji Yamashiro

Abstract

Age-related macular degeneration (AMD) is the leading cause of blindness among elderly people in Western countries. Recently, the prevalence of AMD has also increased in Asia. Although the precise mechanisms of AMD development have not been thoroughly elucidated, both environmental and genetic factors are thought to contribute to its development. As for environmental factors, aging and smoking are the two major risk factors for developing AMD. More than 30 genes associated with AMD have been discovered through genome-wide association studies (GWASs). In addition to their association with the disease development, susceptibility genes for AMD can predict the lesion size and bilaterality of AMD, and genetic information might be useful to conduct personalized medicine for AMD. Recently, the concept of pachychoroid spectrum disease has been introduced, and studies have begun to clarify the genetic architecture of pachychoroid disease.

Keywords

Age-related macular degeneration · Genome-wide association study · Phenotype-genotype association · Personalized medicine
Pachychoroid

6.1 Before GWAS

Genetic linkage analyses discovered genes causing Mendelian macular diseases such as *ABCR* (*ABCA4*) for autosomal recessive Stargardt type 1 macular dystrophy (STGD1) [1], *ELOVL4* for autosomal dominant Stargardt type 3 macular dystrophy (STGD3) [2], *TIMP3* for Sorsby fundus dystrophy (SFD) [3], *EFEMP1* for Malattia Leventinese (ML) [4], and *VMD2* for Best vitelliform macular dystrophy (VMD) [5, 6]. Among these genes, a case-control study reported a possible association between *ABCA4* and AMD in 1997 [7]. However, two later reports from Japan denied such associations [8, 9]. Although a later study from the USA further confirmed associations of *ABCA4* with AMD [10], other studies from various countries denied such associations [11–15]. Considering these previous studies together with recent GWAS reports, *ABCA4* would not be a susceptibility gene for AMD. As for *TIMP3*, two studies evaluated its linkage and association with AMD in 1997 but failed to detect significant roles of *TIMP3* in the

K. Yamashiro (✉)
Otsu Red Cross Hospital, Otsu, Japan
e-mail: yamashro@kuhp.kyoto-u.ac.jp

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development of AMD [16, 17]. However, recent large-scale studies pointed out significant associations of *TIMP3* with AMD. *TIMP3* should be considered a susceptibility gene for AMD. In 1999 and 2000, several studies evaluated associations of *EFEMP1* or *VMD2* with AMD, but these two genes did not show significant associations with AMD [4, 18–20]. The associations between *ELOVL4* and AMD were evaluated in 2001 but *ELOVL4* did not show significant associations with AMD [21]. Although a later study reported significant associations between *ELOVL4* and AMD in 2005 [22], other studies denied such associations [23, 24]. Considering these studies together with the results of recent GWASs, only *TIMP3* would be a susceptibility gene for AMD among causative genes for Mendelian macular diseases (Table 6.1).

Genetic linkage analyses using family data including AMD-affected members discovered several loci for AMD including 1q31 and 10q26 (Table 6.2) [26, 27]. From the 1q31 loci, *HMCN1* was chosen as a candidate gene associated with AMD. In 2003, the first study on *HMCN1* reported its positive associations with AMD in the USA [28]. However, later studies from Finland, the USA, and Japan denied such associations [23, 29–32].

Other genes outside the 1q31 and 10q26 loci were also evaluated for their associations with AMD in candidate gene studies. Since *APOE* had been discovered to have a significant association with Alzheimer's disease in 1993 [33, 34], its association with AMD was evaluated in a case-control study in 1998 [35]. This study revealed significant associations between *APOE* and AMD, and later studies support its association although some studies could not successfully replicate the associations. In East Asians, meta-analysis confirmed significant associations of *APOE* to AMD [36].

Table 6.1 Associations between Mendelian disease genes and AMD

Gene	Mendelian disease	Association to AMD	Confirmed association to AMD in Asian
ABCA4	STGD1	No	No
ELOVL4	STGD3	No	No
TIMP3	SFD	Yes	Yes [25]
EFEMP1	ML	No	No
VMD2	VMD	No	No

AMD age-related macular degeneration, *STGD1* Stargardt type 1 macular dystrophy, *STGD3* Stargardt type 3 macular dystrophy, *SFD* Sorsby fundus dystrophy, *ML* Malattia Leventinese, *VMD* Best vitelliform macular dystrophy

Table 6.2 Genetic linkage analysis results for AMD

Chromosome	Loci
1	1q23.3-q31.1
	1q31.1-q32
2	2p25.1-p23.2
	2p23.2-p16.2
	2p16.2-p12
3	3p25.3-p22.1
	3q22.1-q14.1
	3q12.3-q22.1
4	4q13.3-q24
	4q28.3-q32.1
10	10q23.33-q26.13
	10q26.13-10qter
12	12q23.2-q24.31
16	16p13-q12.2
	16q12.2-q23.1

Since oxidative stress has been thought to play important roles in AMD development and superoxide dismutase has a protective role against oxidative stress, *SOD2* was evaluated for its associations to AMD in Japanese individuals [37]. Although this study reported that *SOD2* was significantly associated with AMD development in 2000, later studies from Japan, North Ireland, and Spain denied such associations [38–40].

Lipid metabolism was also a strong candidate to affect AMD development. Paraoxonase prevents low-density lipoprotein oxidation, and paraoxonase gene polymorphisms have been evaluated for their associations with coronary heart disease and carotid atherosclerosis. In 2001, *PON1* was reported to be associated with AMD in Japanese individuals [41]. Although later studies from Australia, North Ireland, and Spain denied such associations [39, 40, 42], studies from the USA and Turkey supported its association with AMD [43, 44]. The associations between *PON1* and AMD should be further examined.

Since VEGF is a strong promoter of angiogenesis, *VEGFA* has been evaluated in many

genetic studies on AMD. In 2006, a significant association of *VEGFA* and AMD was reported in Caucasians [45, 46]. In 2008, its association with AMD in Chinese individuals was confirmed [47]. In contrast to VEGF, the pigment epithelium-derived factor is a highly effective inhibitor of angiogenesis in cell culture and animal experiments. In 2005, a Japanese group hypothesized a possible association between *PEDF* and AMD [48], and a later study from Taiwan confirmed significant associations between *PEDF* and AMD [49]. However, later studies from Austria, Japan, and China denied such associations and meta-analysis further confirmed that *PEDF* was not associated with AMD [50–54].

6.2 GWAS

The Human Genome Project was completed in 2003 and the International HapMap Project was completed in 2005. Based on these datasets, many GWASs have been conducted for various diseases using single nucleotide polymorphisms (SNPs) as markers. The first successful GWASs of AMD were reported in 2005. Three groups from the USA reported significant associations of *CFH* with AMD [55–57]. Since complement factor H (CFH) is expressed in drusen and regulates complement pathway, it has been speculated that *CFH* affects the development of AMD through inflammatory mechanisms.

The first three studies reported that *CFH* Y402H was the important SNP for AMD, and subsequent studies using Caucasian samples successfully replicated this association. However, many studies from Asia failed to replicate it, possibly due to their small sample size [31, 58–64]. Since the effect allele frequency of the Y402H polymorphism is low among Asians, its association with AMD is hardly detected in studies with small sample size. In 2010, a large-scale study in a Japanese sample successfully reported a significant association of Y402H with AMD, and a meta-analysis also confirmed its significant association in Asians in 2011 [65, 66]. Besides Y402H, the established *CFH* I62V polymorphism is also strongly associated with AMD in both Caucasians and Asians. Since the effect allele frequency is around 60% in Asians, recent studies on *CFH* in Asians mostly evaluate I62V.

The next susceptibility loci for AMD, *ARMS2* (*LOC387715*) and *HTRA1*, were discovered in 2005 and 2006, respectively [67–70]. Since these two genes are located on chromosome 10 within a linkage disequilibrium block, genetic studies have not elucidated which gene is responsible for AMD development. Of the above studies that discovered *ARMS2* or *HTRA1*, one study on *HTRA1* used Chinese samples from Hong Kong while the other three studies used Caucasian samples. After the discovery of these two genes, many studies have successfully replicated the associations of *ARMS2* and *HTRA1* with AMD in both Asians and Caucasians.

In 2010, a GWAS using a Caucasian sample reported a significant association of *LIPC* with AMD together with a weak association of *CETP* and *ABCA1* and a possible association of *LPL* [71]. In the same year, the first meta-analysis of GWASs discovered that lipid metabolism genes such as *TIMP3*, *LIPC*, *CETP*, *LPL*, and *ABCA1* were strongly associated with AMD [72]. However, no Asian samples were included in the discovery samples of this meta-analysis and only 5.6% of the replication samples were of Japanese origin.

In 2013, the largest meta-analysis of GWASs found seven new loci associated with AMD [25]. Furthermore, this study group discovered 16 more susceptibility genes for AMD in 2016 [73]. However, the studied subjects were predominantly of European ancestry, and only 4.3% of the study participants were Asians.

6.3 GWAS in Asia

In 2011, the first GWAS for AMD in Japanese samples discovered two susceptibility loci for AMD: *TNFRSF10A* and *REST-C4orf14-POLR2B-IGFBP7* [74]. Although the association of *REST-C4orf14-POLR2B-IGFBP7* has not been successfully replicated in Asians, the association of *TNFRSF10A* has been confirmed in replication studies in both Asians and Caucasians [25, 75].

In 2013, a GWAS using four Asian populations discovered that an Asian-specific SNP in *CETP* has a strong association with AMD [76]. Furthermore, this study discovered three new loci for AMD: *C6orf223*, *SLC44A4*, and *FGD6*. A later study revealed that *FGD6* is a susceptibility gene for only polypoidal choroidal vasculopathy (PCV) but not AMD without polypoidal lesions [77].

6.4 Candidate Gene Analysis

After the discovery of *CFH*, several candidate genes in the complement pathway were examined for their possible associations with AMD in case-control studies. Among them, *C2/CFB* was reported as a susceptibility gene for AMD in 2006 [78], *C3* was reported as a susceptibility gene for AMD in 2007 [79, 80], and *CFI* was reported as a susceptibility gene for AMD in 2009 [81]. All four loci have been confirmed to have significant associations with AMD in Asians as well. *C5* was also examined for its association with AMD in 2007 but it did not show significant association [79, 80]. In 2008, a case-control study proposed *SERPING1* (*C1IN*) as a susceptibility gene for AMD [82]. However, later studies denied such associations in both Asians and Caucasians [83–85]. Meta-analysis also denied associations between *SERPING1* and AMD in Asians [86].

6.5 Confirmed Association to AMD in Asians

So far, susceptibility genes for AMD only in Asians or only in Caucasians have not been discovered, suggesting that all AMD susceptibility genes discovered in Caucasians would be also susceptibility genes for AMD in Asians and vice versa. Susceptibility genes for AMD are summarized in Table 6.3. The confirmation of the association with AMD in Asians is also noted. Genes with genome-wide association with AMD in at

Table 6.3 AMD susceptibility genes with genome-wide significance

		Confirmed association to AMD in Asian	
Chromosome	Gene	Original study	Replication by other studies
1	*CFH*	Caucasians only [55–57]	Yes [25, 76]
2	*COL4A3*	Data unavailable [73]	Yes [87]
3	*ADAMTS9*	Yes [25]	Yes [76]
3	*COL8A1/FILIP1L*	No [25]	
4	*CFI*	Caucasians only [88]	Yes [25, 76]
5	*C9*	Data unavailable [73]	
5	*PRLR/SPEF2*	Data unavailable [73]	
6	*IER3/DDR1*	Yes [25]	
6	*C2/CFB*	Caucasians only [78]	Yes [25, 76]
6	*VEGFA*	Caucasians only [45, 46]	Yes [25, 76]
6	*FRK/COL10A1*	Caucasians only [89]	Yes [25]
7	*PILRB/PILRA*	Data unavailable [73]	
7	*KMT2E/SRPK2*	Data unavailable [73]	
8	*TNFRSF10A*	Yes [74]	Yes [25, 75]
9	*COL15A1/TGFBR1*	Yes [25]	Yes [76]
9	*TRPM3*	Data unavailable [73]	
9	*MIR6130/RORB*	Data unavailable [73]	
9	*ABCA1*	Data unavailable [73]	Yes [90]
10	*ARMS2/HTRA1*	*ARMS2*: Caucasians only [67, 70]	Yes [25, 76]
		HTRA1: Yes [68]	
10	*ARHGAP21*	Data unavailable [73]	

Table 6.3 (continued)

Chromosome	Gene	Confirmed association to AMD in Asian	
		Original study	Replication by other studies
12	*RDH5/CD63*	Data unavailable [73]	
12	*ACAD10*	Data unavailable [73]	
13	*B3GALTL*	Yes [25]	
14	*RAD51B*	Yes [25]	Yes [91]
15	*LIPC*	No [72]	Yes [25]
16	*CETP*	Yes [72]	Yes [76]
16	*CTRB2/CTRB1*	Data unavailable [73]	
17	*TMEM97/VTN*	Data unavailable [73]	
17	*NPLOC4/TSPAN10*	Data unavailable [73]	
19	*C3*	Caucasians only [79]	Yes [92, 93]
19	*APOE*	Caucasians only [94]	Yes [25, 76]
19	*CNN2*	Data unavailable [73]	
20	*MMP9*	Data unavailable [73]	
20	*C20orf85*	Data unavailable [73]	
22	*SYN3/TIMP3*	No [72]	Yes [25, 76]
22	*SLC16A8*	Yes [25]	

least one study and with confirmed association with Asian AMD in at least two studies are *CFH*, *ADAMTS9*, *CFI*, *C2/CFB*, *VEGFA*, *TNFRSF10A*, *COL15A1/TGFBR1*, *ARMS2/HTRA1*, *RAD51B*, *CETP*, *C3*, and *APOE*.

6.6 New Treatments

After the discovery that complement pathway genes were important susceptibility genes for AMD, many complement pathway-related drugs have been tested to treat AMD. Eculizumab, an antibody to block C5, is an intravenous drug to treat paroxysmal nocturnal hemoglobinuria. Although eculizumab was tested for eyes with drusen or geographic atrophy (GA), it did not decrease drusen or prevent GA growth in phase 2 clinical trial. LFG316, another antibody against C5, was also tested for patients with dry AMD. However, the monthly intravitreal injection of LFG316 did not show significant preventive effects on GA in phase 2 clinical trial. Zimura (ARC1905) is an aptamer against C5. A phase 2/3 clinical study is now evaluating its effects on GA. Compstatin derivatives POT-4 and APL-2 are inhibitors of C3. Both drugs are injected intravitreally and clinical trials of APL-2 are now ongoing for GA. Lampalizumab, an antigen-binding antibody fragment against complement factor D (CFD), showed significant preventive effects on GA progression in the MAHALO phase 1/2 study. However, a phase 3 study did not validate its suppressive effects on GA progression. In addition to these drugs, several drugs in the complement pathway have been tested for AMD treatment (Table 6.4).

Table 6.4 Complement pathway-related drugs for AMD

Drugs	Mechanism of action
Eculizumab	Antibody to block C5
LFG316	Antibody to block C5
Zimura (ARC1905)	Aptamer against C5
JPE1375	Antibody against C5a receptor
JSM-7717	Antibody against C5a receptor
POT-4 (AL-78898A)	Compstatin derivative, C3 inhibitor
APL-2	Compstatin derivative, C3 inhibitor
Lampalizumab (FCFD4514S, RG7417)	Fab fragment of antibody against CFD
BXC1470	CFD inhibitor
TA106	Fab fragment of antibody against CFB

Although *ARMS2* has a strong association with AMD development, the location of its expres-

sion has not been determined and its role in the development of AMD has not been elucidated. Its expression in the human retina was confirmed by PCR when its association with AMD was discovered [70], and its localization to the mitochondrial outer membrane was demonstrated with immunocytochemistry using COS-1 cells in 2007 [95]. In 2008, immunostaining of the human retina showed localization of ARMS2 protein in the mitochondria of photoreceptor cells [96]. However, in 2010, an immunocytochemistry study using ARPE-19 cells reported localization of ARMS2 to the endoplasmic reticulum and an immunostaining experiment demonstrated secretion of ARMS2 protein to the extracellular matrix around the capillaries of the choroid, with the highest concentration adjacent to Bruch's membrane [97]. This study reported only faint and diffuse ARMS2 staining in the retinal pigment epithelium (RPE) and the retina. In 2010, its expression in human blood was also confirmed by PCR [98]. A recent study demonstrated that ARMS2 is expressed in monocytes and resident retinal microglia [99]. This study proposed that ARMS2 protein secreted by microglial cells would bind to glycosaminoglycans on the surface of late apoptotic or necrotic cells to enhance complement activation and assist in the clearance of cellular debris in the human retina, which can prevent the formation of drusen. Since the role of ARMS2 has not been elucidated in the development of AMD, drugs targeting ARMS2 have not been tested to treat AMD.

HTRA1 is a serine protease. Animal experiments have shown that overexpression of HTRA1 in the RPE leads to ultrastructural changes in the elastic layer of Bruch's membrane via cleavage of extracellular matrix components [100] and induces the development of choroidal neovascularization (CNV) [101]. However, no drug has been developed for AMD by targeting HTRA1.

6.7 AMD Subtypes

Although AMD susceptibility genes do not show clear differences among ethnicities, some genes might be able to explain the differences between AMD subtypes. Neovascular AMD can be divided into three subtypes: polypoidal choroidal vasculopathy (PCV), retinal angiomatous proliferation (RAP), and typical AMD. The association strength of *ARMS2* was found to differ among these three subtypes of Japanese AMD in 2010 [65]. It was strongest for RAP, intermediate for typical neovascular AMD, and weaker for PCV. The reported risk allele frequencies of the *ARMS2* A69S polymorphism were about 40% in normal controls, while they were 55% in PCV, 65% in typical neovascular AMD, and 90% in RAP. *ARMS2* might contribute to the difference in the development of these subtypes.

In 2008, *ELASTIN* polymorphism was reported to be associated only with PCV but not with typical AMD in Japanese individuals [102]. Since elastin is an important component of Bruch's membrane and its function as a barrier to vessel growth, the integrity of Bruch's membrane might be able to explain the difference between typical AMD and PCV. However, later Japanese studies with larger sample sizes denied associations of *ELASTIN* with PCV [103, 104]. On the other hand, *CD36* polymorphism was reported to be associated only with typical AMD but not with PCV [105]. However, no replication study has confirmed such associations. *FDG6* is also a possible gene differentiating PCV from typical AMD. In 2016, a Chinese study discovered a rare variant in *FDG6* that was significantly associated with PCV but not with typical AMD [77]. This finding was successfully confirmed in Chinese, Japanese, and Singaporean samples.

As for the difference between neovascular AMD and dry AMD, *TLR3* was proposed as a susceptibility gene for dry AMD in 2008 [106]. However, later studies denied such associations in both Asians and Caucasians [86, 107–109]. Among the known susceptibility genes for AMD, the four loci of *ARMS2/HTRA1*, *CETP*, *MMP9*, and *TIMP3* showed significant differences in their association between wet AMD and dry AMD [73]. The effects of *ARMS2/HTRA1*, *CETP*, and *TIMP3* were stronger for wet AMD than for dry AMD, while *MMP9* might be a susceptibility gene only for wet AMD.

6.8 Phenotype-Genotype Associations

Associations between AMD susceptibility genes and the bilaterality of AMD have been evaluated in many studies. As for *ARMS2/HTRA1*, the odds ratio of its association with AMD development was reported to be higher for bilateral AMD than for unilateral AMD in 2007 and 2009 [110, 111]. In 2008, significant differences in the genotype distribution were reported between bilateral AMD and unilateral AMD [111, 112]. Also in PCV, significant differences in the genotype distribution were reported between bilateral patients and unilateral patients [113]. In 2017, a GWAS further confirmed a strong association of *ARMS2/HTRA1* with the bilaterality of AMD in Japanese individuals [114].

In a hospital-based study using more than 300 Japanese patients with unilateral AMD, it was demonstrated that genetic information about *ARMS2/HTRA1* could predict the development of AMD in the fellow eye [115]. Only around 10% of the patients with the GG genotype in the *ARMS2* A69S polymorphism developed AMD in the fellow eye during a follow-up period of more than 10 years, while around 50% of the patients with the GT genotype developed AMD in the fellow eye. In the patients with the TT genotype, the fellow eye developed AMD around 70% of the time. A genetic risk score using *ARMS2/HTRA1*, *CFH*, *TNFRSF10A*, *VEGFA*, and *CFI* gene polymorphism information would be more useful to predict AMD development in the fellow eye. When a genetic risk score was calculated for 891 Japanese patients with unilateral AMD, the score showed a strong association with the 10-year incidence of AMD in the fellow eye [116]. Although a large prospective study denied associations between AMD susceptibility genes and the bilaterality of AMD [117], genetic information could be useful to predict fellow eye development of AMD.

Although some studies denied associations between *ARMS2/HTRA1* and CNV size in AMD, many studies have reported positive associations of *ARMS2/HTRA1* with CNV size in AMD or PCV [112, 118, 119]. In 2015, a GWAS from Japan confirmed genome-wide level associations between *ARMS2/HTRA1* and CNV size in AMD [120]. Genetic information about *ARMS2/HTRA1* would be useful to predict the lesion size of neovascular AMD. Besides *ARMS2/HTRA1*, associations of *CFH* with the bilaterality of AMD or with the CNV size in AMD have also been evaluated, but many studies failed to detect significant associations of *CFH*. *CFH* does not appear to strongly affect the bilaterality or CNV size of AMD.

In addition to fellow eye involvement and lesion size, *ARMS2/HTRA1* might be able to predict subretinal hemorrhage and vitreous hemorrhage in eyes with PCV. One Japanese group reported two studies showing that the AMD risk allele for that locus was significantly associated with vitreous hemorrhage [121] or subretinal hemorrhage and hemorrhagic retinal pigment epithelium detachment [113].

6.9 Personalized Medicine

Genetic information has been used for personalized medicine to treat various diseases. To predict treatment responses for AMD, *ARMS2/HTRA1* might be useful. For visual prognosis after photodynamic therapy (PDT) for AMD, three studies from Japan reported a significant association of *ARMS2/HTRA1* (Table 6.5) [119, 122, 123]. Patients with risk alleles for AMD tended to show worse visual outcomes after PDT, while patients with protective alleles for AMD tended to show improvement of visual acuity after PDT. In contrast, two reports on Caucasian AMD did not show such associations of *ARMS2/HTRA1* with visual outcome after PDT [124, 125]. In addition to *ARMS2/HTRA1*, other genes such as *VEGFA*, *PEDF*, and *CRP* were evaluated for their associations to the treatment outcome after PDT for AMD. Further study is needed for these genes, and further confirmation is needed to use genetic information about *ARMS2/HTRA1* for personalized medicine to treat AMD with PDT.

In contrast to PDT, study results on predictive genes for treatment outcomes after anti-VEGF treatment are still controversial. Among estab-

Table 6.5 Association of *ARMS2/HTRA1* with visual prognosis after photodynamic therapy for age-related macular degeneration

	N	Ethnicity	Subtype	Association
Chowers et al. [124]	139–143	Caucasian	AMD	No
Brantley et al. [125]	32	Caucasian	AMD	No
Sakurada et al. [122]	71	Japanese	PCV	Yes
Tsuchihashi et al. [123]	110	Japanese	tAMD/PCV	Yes
Besssho et al. [119]	68	Japanese	tAMD	Yes
	119	Japanese	PCV	Yes

lished susceptibility genes for AMD, the three genes *CFH*, *ARMS2/HTRA1*, and *VEGFA* have been evaluated in many studies. As for *CFH*, the first study reported its significant association with visual outcome after anti-VEGF treatment for AMD [118]. Although several later studies have reported positive associations of *CFH* with the treatment outcome after anti-VEGF treatment, many studies from Asian countries denied such associations [126–131]. Considering that the largest prospective study with more than 800 participants also denied such associations of *CFH* [132], genetic information about *CFH* may not be useful for personalized medicine to treat AMD with anti-VEGF drugs.

For *ARMS2/HTRA1*, the first study reported its significant association with visual outcome after anti-VEGF treatment for AMD [133], and several later studies support such associations [129, 134, 135]. In contrast, several studies from Asia have denied the possibility of *ARMS2/HTRA1* as a predictive gene for visual outcome after anti-VEGF treatment for AMD [126, 127, 130, 131]. Recently, a prospective multicenter study was performed in Japan on genome-wide associations with the anti-VEGF treatment outcome for AMD [136]. Although this GWAS could not find genes with genome-wide level association with visual outcome after anti-VEGF treatment, it confirmed that *ARMS2/HTRA1* was significantly associated with an additional treatment requirement in the prospective study cohort with 461 Japanese patients with AMD. *ARMS2/HTRA1* might be useful for personalized medicine to treat AMD with anti-VEGF drugs.

6.10 Genes Associated with Pachychoroid Diseases

Late AMD usually develops from early AMD with drusen. However, many Asians develop late AMD without drusen. The mechanisms of developing late AMD without drusen were unclear until the recent proposal of a pachychoroid disease spectrum. Pachychoroid means thick choroid, and it is well known that optical coherence tomography images of eyes with central serous chorioretinopathy (CSC) show pachychoroid. In addition to CSC, pachychoroid induces damage of the RPE, resulting in pachychoroid pigment epitheliopathy [137]. Furthermore, pachychoroid induces GA or CNV, resulting in pachychoroid geographic atrophy and pachychoroid neovasculopathy, respectively [138, 139].

Although the genetic architecture of pachychoroid geographic atrophy and pachychoroid neovasculopathy have not been clearly elucidated, the genetic architecture of pachychoroid itself was recently revealed by a GWAS in a

Japanese population; *CFH* and *VIPR2* showed strong associations with choroidal thickness [140]. Interestingly, risk alleles of *CFH* for AMD development worked protectively against choroidal thickening, while the protective alleles for AMD development tended to make the choroid thinner (Fig. 6.1).

Genetic background studies are so far limited for pachychoroid geographic atrophy and pachychoroid neovascularization. In Caucasians, the frequency of the risk allele in *CFH* for AMD was reportedly about 63% in AMD, 46% in pachychoroid neovasculopathy, about 31% in controls, and about 24% in pachychoroid without CNV (Table 6.6) [141]. In Japanese individuals, the frequency of the risk allele in *CFH* for AMD was reportedly about 75% in AMD, 59% in pachychoroid neovasculopathy, about 59% in controls, and 46–59% in pachychoroid without CNV [142]. In Caucasians, the risk allele frequency in pachychoroid neovasculopathy falls between those of AMD and controls. However, the risk allele frequency in pachychoroid neovasculopathy was similar to controls in Japanese individuals. This difference might be an ethnic difference in the causation of pachychoroid neovasculopathy or it might be explained by the difference in definition of pachychoroid neovasculopathy between two studies.

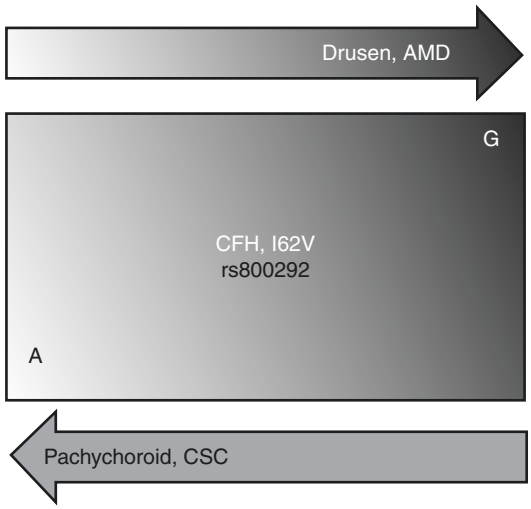

Fig. 6.1 Associations of *CFH* I62V polymorphism. G allele is associated with development of drusen and age-related macular degeneration (AMD) while A allele is associated with development of pachychoroid and central serous chorioretinopathy (CSC)

As for the *ARMS2* A69S polymorphism, both Caucasian and Japanese individuals showed similar results (Table 6.7). The frequency of its risk allele for AMD was highest in AMD, higher in pachychoroid neovasculopathy, and lower in pachychoroid without CNV.

Since drusen-related AMD and pachychoroid neovasculopathy belong to genetically different clinical entities, future studies may achieve better detection by distinguishing between cases of drusen-related AMD and pachychoroid neovasculopathy. Further genetic studies might enable us to use personalized medicine for drusen-related AMD and pachychoroid neovasculopathy.

Table 6.6 *CFH* risk allele frequencies for age-related macular degeneration in pachychoroid diseases

Caucasian	AMD	PCN	GA	PGA	Control	Pachychoroid
Y402H, C	0.63	0.46			0.31	0.24
Japanese	AMD	PCN	GA	PGA	Control	Pachychoroid
I62V, G	0.75	0.59	0.77	0.71	0.59	0.46–0.59

AMD age-related macular degeneration, *PCN* pachychoroid neovasculopathy, *GA* geographic atrophy, *PGA* pachychoroid geographic atrophy

Table 6.7 *ARMS2* risk allele frequencies for age-related macular degeneration in pachychoroid diseases

Caucasian	AMD	PCN	GA	PGA	Control	Pachychoroid
A69S, T	0.44	0.41			0.22	0.15
Japanese	AMD	PCN	GA	PGA	Control	Pachychoroid
A69S, T	0.60	0.51	0.69	0.32	0.39	–

AMD age-related macular degeneration, *PCN* pachychoroid neovasculopathy, *GA* geographic atrophy, *PGA* pachychoroid geographic atrophy

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7 The Contribution of Rare Allele and Junk Genome in AMD Pathogenesis

Kaushal Sharma, Rahul Tyagi, Priya Battu, Saurabh Kumar, Neel Kamal Sharma, and Akshay Anand

Abstract

The analysis of degenerative diseases has revealed genetic heterogeneity and this appears to contribute towards an increase in the complexity of disease with influences from environmental factors. Worldwide Genetic studies have identified various common and non-synonymous alleles with AMD across the geographically distributed population. With the advancement in technology, the biological contribution of rare alleles and "Junk DNA" in AMD has been suggested. The biological significance of intronic, rare alleles, and transposons in degenerative diseases can provide the necessary information to assess the evolution of the complexity of AMD pathology.

Keywords

Age-related macular degeneration · Rare allele · Junk DNA · Retrotransposon · Alu

7.1 Biological Significance of Rare Alleles and Junk Genome

The recent era is characterized by sophisticated technologies (including Next Generation Sequencing and Whole Genome Sequencing) in the field of Genetics. This can enable screening of a broad spectrum of genomic variations through population-based studies. The biological significance of rare allele (q allele <0.01) was difficult to predict in disease pathology due to lack of evidence and number of participants in the study. Rare alleles can be assessed by employing the imputation, genotype, and GWAS strategies on huge sample size. Rare allele analysis and its penetrance could be further dissected by various statistical measures including burden (ARIEL, KBAC, EREC, etc.), combined (e.g., EMMPAT, SKAT-O, MiST, etc.), variance-component tests (KBAT, SSU, SKAT, C-Alpha, etc.), least absolute shrinkage and selection operator (LASSO) and exponential combination (EC) tests [1]. A report from *Science* (2012) has suggested the population explosion (from millions to >7 billion) over the periods of 10,000 years and was in

K. Sharma · R. Tyagi
Department of Pediatrics, Allergy and Immunology Lab, Post Graduate Institute of Medical Education and Research, Chandigarh, India

P. Battu · S. Kumar · A. Anand (✉)
Department of Neurology, Neuroscience Research Lab, Post Graduate Institute of Medical Education and Research, Chandigarh, India
e-mail: anand.akshay@pgimer.edu.in

N. K. Sharma
Armed Forces Radiobiology Research Institute, Bethesda, MD, USA

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concordance with a drastic increase in rare alleles and gene variants throughout the human genome. Exponential increase in population growth can enhance the chance of mutation accumulation and rare allele burden in the human genome and could also stimulate the complex disease phenotypes [2]. Though naturally selected rare allele variants did not reveal direct evidence to ascribe for disease pathology yet it could increase the risk of disease by supporting the genetic susceptibility hypothesis. Interestingly, rare allele of CFH variant (P503A) could act as a signature to stratify the AMD patients from the Amish population as compared to non-Amish to accommodate for the environmental changes [3]. Noteworthy increase in the number of rare alleles may be derived due to functional changes in repair mechanisms (supporting natural selection of variants), generation of the new hot spot for genetic mutation as a part of evolution (environmentally stimulated) and may even cross the threshold of genetic variations in the genome and perhaps reflect as disease phenotypes. With the growing number of publications in AMD genetics, various rare alleles have been discovered over time. Fritsche et al (2015) have identified the rare allele (<0.1%) of *CFH, TIMP-3,* and *CFI* in the exome region of genes in AMD. Results also demonstrated the genetic variation at the splicing site of *SLC16A8*. Such genomic variations can systematically classify the genetic variation in particular disease which needs an extremely large population size [4]. A rare allele penetrance of CFH c.3628C>T mutation is also found to confer the risk of AMD [5]. p.Lys155Gln non-synonymous substitution of C3 has been derived from rare allele variation in AMD patients which showed a reduced binding affinity with CFH molecule in the downstream alternative complement cascade and contributed in the pathology [6]. Additionally, AMD has also been associated with other rare genetic variants investigated in complement factors including missense CFI, p.Lys155Gln of C3 and p.Pro167Ser of C9 [7]. Some of the other rare alleles have also been discussed in Table 7.1.

Initially, most of the GWAS came to a conclusion of "common disease-common variant" (CD-CV or missing heritable problem) hypothesis with most the pathologies studied over the globe do not signify the complexity characteristic. Of degenerative diseases including AMD. However, how a few dozen variants exhibit a moderate effect on disease pathology with intermediate frequency can be argued in population genetics-based studies. In addition to CD-CV, identified genetic loci or component can be designated in one of the following class, e.g., infinitesimal (common variants with large frequency and small-effect), rare allele (rare variants with large number and large-effect), and broad-sense heritability (combination of genetic, epigenetic, and environmental interactions) models which can be justified with the heterogeneity of degenerative diseases [18].

In addition to the base substitutions, transposable elements (TEs) are major mutations that contribute towards genome evolution. TE not only affects the structure of the genome but alters the expression of various proteins as well [19]. During the course of evolution, TEs have been naturally selected as a robust source of regulatory sequences pertaining to organism's development and functions. Dysregulation of these sequences can apparently cause many diseases like Cancer and autoimmunity [20]. Transposon-based insertional mutagenesis (TIM) is used to create mice models for cancer and also being utilized for identifying cancer-causing genes [21]. Jiang et al have shown that transposons provide sites for the binding of transcriptional factors in breast cancer and also contain vast promoter activity. TEs are also implicated in many neuropsychiatric brain disorders. TEs are responsible for causing insertions and deletions as well as copy number variants (CNVs) in the genome of the brain resulting in autism, schizophrenia, Alzheimer's disease (AD), etc. [22]. Autism is one such disorder characterized by variety of impairments in behavioral, cognitive, social, and communicative patterns. About 10% of autism cases have been reported due to chromosomal aberrations, non-allelic homologous recombination (NAHR), non-homologous end-joining, break-point induced replication and transposon-mediated events [23]. Prudencio et al. have exhibited the higher expression of repetitive sequences like

Table 7.1 Rare alleles and genetic loci of various genes identified in AMD and their clinical correlation in the widely distributed demographic population

S. No.	Gene	Rare allele	loci	Population	Year	Clinical impression	References
1.	*CFH*	C>T	rs121913059	Western USA, eastern USA, Britain, western Europe, and Australia	2018	–	[8]
2.	*CFH*	C>T	rs35292876				
3.	*CFH*	T>A,C	rs183474263	European	2015	Involving in CFH cofactor activity	[9]
4.	*C3*	T>G	rs147859257	USA	2013	–	[10]
5.	*TNFRSF10A*	G>A,C,T	rs13278062	Japan	2011	New susceptibility locus for exudative AMD	[11]
6.	*REST-C4orf14-POLR2B-IGFBP7*	G>T	rs1713985				
7.	*C9*	G>A	rs147701327	European	2016	–	[12]
8.	*CFI*	G>C	rs146444258	USA	2018	This missense variant is associated with early onset of disease	[13]
9.	*C2*	G>A,C	rs9332739	USA	2006	Confers reduced risk of AMD	[14]
10.	*BF*	T>A	rs4151667	USA	2006		
11.	*PELI3*	C>T	rs145732233	European	2016	Associated with decreased risk of disease	[15]
12.	*ACKR3*	C>T	rs56072732	USA	2018	Marginally associated with disease progression	[16]
13.	*HERC1*	T>C,G	rs74320127				
14.	*POC5/SV2C*	T>C	rs79069165				
15.	*MMP9*	TTTCTTTCTTTCT>TTTCTTTCT,TTTCTTTCTTTCT	rs142450006	USA Caucasians	2018 2019	Associated with CNV not GA Not known	[17]
16.	*LCN9*	A>G	rs200437673	Amish			
17.	*RTEL1*	G>A	rs151214675	Population			
18.	*DIGAP1*	A>G	rs140250387	USA			
19.	*CGRRF1*	G>A	rs115333865				

long interspersed elements retroviruses and DNA transposons in brains especially the frontal cortex in *C9orf72* Amyotrophic Lateral Sclerosis (ALS) patients. The expression was significantly higher *C9orf72* patients as compared to non *C9orf72* or normal controls [24]. Conclusive statement based on results of studies suggesting the imperative role of such genetic variants under the influence of confounders, differential demographic distribution, and environmental factors prevail in the particular population. Hence, it is imperative to investigate the functional and pathological significance of such genetic variations and "*junk DNA*" in the evaluation of genetic and disease complexity.

7.2 Mutation Threshold and Functional Diversification

Evolution is a persisting and diversification process which has led to the creation of new species based on dynamic environmental changes. However, evolution is initiated on micro- and macromolecular levels and gets accumulated over a period of time which can be reflected in species development as well as at the complexity of disease phenotypes in the modern era. Various diseases including AD, Cancer, AMD, etc. have exhibited the complexity not only based on their disease phenotype but also based on their genetic hetero-complexity which is found to increase drastically in recent times. The mutation threshold of mitochondrial gene *MTND1* can lead to the performance of the dual functions including pro- and anti-tumorigenic (called *oncojanus*) to adapt against HIF-1α mediated mechanism. Hetero or homoplasmy status can also affect the tumorigenic and metastatic condition of cells which suggests the accumulation of mutation in the mitochondrial and nuclear genome as per the changes in the micro-environment of the cell [3]. Moreover, the mitochondrial genomic variation can also modify the function of respiratory complex I concomitant with the tumor progression, suggesting the directional selection of mutation in the genome [25]. Macular RPE cells collected from AMD patients have shown the accumulation of heteroplasmic mutation with reduced repair mechanisms [26, 27]. Surprisingly, co-occurring genomic variation in non-small-cell lung cancer also indicates the accumulation of genetic alterations as a result of the change (genes including *TPS2, STK11*) in micro-environmental vicinity of the cell which could alter the therapeutic strategies of an individual [28]. In germinal center B-cell, both activation-induced cytidine deaminase (AID) and ATM can sense and regulate the mutation threshold and immune diversification during B-cell maturation [29]. Conclusively, the results of the studies suggest the adaptive specification and diversification of various characters and functions based on the micro-environment and alterations in cellular properties. Changes in the genome may accumulate over the period of time which may further lead to the development of complex traits and diseases based on developed genetic interactions and penetrance in the subsequent progeny. More studies are warranted in the field of such variations to examine genetic and pathological complexities of various degenerative diseases including AMD with evolutionary changes over the period of time.

7.3 Evolutionary Impact of Junk Genome in Complexity of Degenerative Diseases

Transposable elements (TEs) have been implicated and found to play a crucial role in evolutionary process, maintenance of genomic architecture, governing regulatory mechanisms and contributes in the normal as well as diseased condition. TEs make up two-third of the entire human genome and are considered to be low complexity elements including Long Interspersed Nuclear Elements (LINEs), Short Interspersed Nuclear Elements (SINEs), and human endogenous retrovirus (HERVs). The literature review suggests increased expression of certain LINE, SINE, and LTR in different neurodegenerative diseases [30]. Although a significant amount of work has already been done, still its considerable role in the genome evolution, progression and

development of human health and neurodegenerative disease is not adequate [31]. In early 1988, a group of researchers demonstrated de novo insertion of TE in hemophilia A for the first time [32]. Genomic projects including Encyclopedia of DNA Elements (ENCODE) and Functional Annotation of Mouse (FANTOM) have suggested the cell-specific expression of TE which controls self-cell specific transcription [30]. It is evident that retrotransposons can produce neural somatic assortment [33]. In terms of evolution, L1s are considered as one of the most ancient TEs in eukaryotes. Human genome contains many inserted active variants of TEs, like *Alu* insertions (*AluYa5, AluYb8, AluYc1*). In most the cases, TEs having insertions and excisions which are responsible for genomic instability, which may cause variety of diseases including neurodegenerative disorders. Around 0.3% of mutations associated with TEs are caused by insertions. These mutations are deleterious in the sense that it can disrupt the active sequence of a functional gene. If these inserts are occurring within an exonic region, it generally changes the ORF, in a way that it codes for an abnormal peptide, and if the insertions occur within the intron, it may lead to an alternative splicing [34].

Recent study has shown the expansion of SINE (especially SINE B2) mediated by ChAHP (CHD4, ADNP, HP1) complex and competes with CTCF (key regulator of chromatin structure). Results indicate the evolutionary significance of such a mechanism in the functional and structural diversification of an organism [35]. Importantly, the described role of mobile elements in lifestyle-induced oxidative burden is still a topic of future studies. Giorgi et al. reported that oxidative damage affects LINE-1 retrotransposition in human neuroblastoma cells [36]. In such a situation, a lifestyle and demographic distribution based increase in oxidative burden may result in genetic complexity and instability of the human genome which may mediate through SINEs and LINEs mobile activity or cellular toxicity. It is pertinent to note that the human genome has been preserved in terms of molecular changes, throughout evolutionary phases. However, recent changes in lifestyle may trigger an unexpected increase in the disease burden. Similarly, allostatic stress exposure can also alter the copy number of retrotransposon and its activity can provide the wear and tear state of cell. Interesting, copy number and activity of retrotransposon can vary among different tissues which may be depend on their uses and micro-environment alteration in a particular tissue. This may help us to understand that some tissues are more susceptible for the disease under the influence of stressors and some are not [37]. Interesting, long, and repeated sequences in the neuronal cell can promote the biosynthesis of cirRNA under the limited of spliceosome activity [38]. The study is indicating genome complexity can induce the phylogenetic along with functional diversification consequently can lead to distinct levels of complexities and the genesis of diseases based on that.

7.4 Junk Genome and Biological Significance in AMD

Alu transposons are DNA sequences, considered as non-coding DNA that can change their positions, create mutations, alter genome size, and even lead to disruption in genetic material [39, 40]. These elements are considered as "*Junk DNA*," although these are hot spots and landscape formative gear of our genome which can affect our health through self-propagation and accumulation. *Alu* transposons are involved in many neurodegenerative diseases and can produce mutations by inserting within or regulatory sequences in the vicinity of genes. Many inherited disorders are resultant from *Alu* insertions. In age-related macular degeneration (AMD), transposons play an important role. It has been previously shown that the decreased expression of DICER1 in retinal pigment epithelium (RPE) of humans and its conditional ablation induces RPE degeneration. The neuronal connection of TEs indicates the regulatory role in various neurodegenerative diseases including AMD. Recently, Maugeri et al demonstrated to enhance the activity of DNMTs in AMD post-mortem choroid and RPE samples. LINE-1 levels were found to be enhanced in AMD cases in comparison to the

controls. These results are in line with the previous reports in AD [41]. LINE-1 retrotransposon accounts for approximately 17% of the human genome. Similarly, SINEs have also been implicated in the pathophysiology of AMD. Gelfand et al reported SINEs mediated transcription of RNAs in the presence of increased iron [42]. These sites were also targeted to slow down or reduce the degeneration processes and enhancement of RPE function. Iron toxicity-associated SINE RNA accumulation is regulated by suppression of DICER1 [42]. Deficits in DICER1, in turn, leads to activation of the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3) inflammasome and amplification of IL-18. This mechanism can further activate the caspase-8 induce apoptotic pathway resulting in the degeneration of RPE cells in AMD cases with geographic atrophy [43]. The advanced AMD stage, i.e., geographical atrophy has remained an untreatable condition and results in blindness. Caspase-8 mechanism is thought to be a potential target to cure advanced stages of AMD.

The activity of retrotransposons is also evident in many retinal disorders like Age-related macular degeneration. Kaneko et al. showed that DICER 1 (miRNA digesting enzyme) is responsible for degrading *Alu* elements, accumulation of which leads to GA like pathology in RPE in DICER knockouts. This shows that DICER is involved in the degradation of retrotransposons which may cause blindness in humans [40]. Kim et al further revealed that *Alu* RNA is also responsible for activation of caspase-8 downstream of NLRP3 inflammasome resulting in RPE degeneration and can rescue the same by inhibiting caspase-8. This suggests that apoptosis plays role in degeneration induced by *Alu* RNA toxicity [43]. A study on mice has demonstrated that iron overload causes RPE degeneration via the inflammasome pathway which can further induce accumulation of SINE due to decreased activity of DICER-1 and can lead to degeneration of RPE [42]. Studies have shown the involvement of *NF-κB* and *P2X7* in conjecture with *Alu* elements responsible for RPE degeneration [44]. A study by Chong et al using ARPE 19 cell culture system showed that pretreatment of APRE 19 cells with Lutein and in combination with zeaxanthin improves the viability of ARPE-19 cells and decreased levels of *Alu* RNA transcripts showing the role of *Alu* elements in the survival of ARPE-19 cells [45].

DICER1 is involved in the degradation of *Alu* RNAs and undigested *Alu* RNAs can induce AMD phenotype in mice following direct cytotoxicity to retinal pigment epithelium (RPE) cells [40]. However, there are many questions related to the mechanism of *Alu* RNA accumulation in AMD patients. It is hypothesized that heat shock, oxidative stress or viral infection, etc. under the influence of senescence and aging, can induce *Alu* RNA accumulation [46–48]. The RNAs transcribed from *Alu* in rodents have demonstrated complex regulatory functions such as modulation of alternative splicing and transcriptional repression [49–51]. Reports have also shown significant genetic diversity in *Alu* polymorphism of the human population [52]. Moreover, silencing of toxic *Alu* transcript has elucidated a critical cell function for DICER1 and results have revealed the activation of extracellular-signal-regulated kinase (ERK) "classical mitogen-activated protein kinases (MAPK)"—as key mediators of Alu RNA accumulation or DICER1 dysregulation-induces RPE cell death in AMD [53]. Targeting therapies like anti-sense oligo-nucleotides (ASON) prevent *DICER1* reduction and inhibit RPE degeneration despite other miRNA down-regulation [40]. The capability of *Alu* RNA-antisense oligonucleotide to prevent DICER1 depletion has been found to induce RPE cytotoxicity and delivered a rationale to further investigate the *Alu* RNA or DICER1 based therapies in AMD. However, still the mechanism of *Alu* RNA cytotoxicity and downstream signaling cascades are not fully defined.

7.5 Conclusion

Association of rare allele could significantly assess the phylogenetic evolution and genetic complexity of heterogenic AMD which may account for a causal role of variants by Mendelian Randomization (MR) in disease. Development

of rare alleles and propagation of repetitive sequences and "*junk genome*" (*Alu* and *SINE* sequences) throughout the genome are suggestive of the complex nature of genetic interactions and pathological developments under the influence of particular environment influence. Contribution of rare allele and "*junk DNA"* in AMD genetics can provide the precise genetic diagnostic and therapeutic target to deal pathology.

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8 Differential Genotypes in Age-Related Macular Degeneration and Polypoidal Choroidal Vasculopathy: A Updated Meta-Analysis

Haoyu Chen, Li Ma, Xulong Liao, Li Jia Chen, and Chi Pui Pang

Abstract

Neovascular age-related macular degeneration (nAMD) and polypoidal choroidal vasculopathy (PCV) have some shared risk factors and clinical manifestation, but there are also some different features. Genetic variants are an important risk factor for both conditions. In this chapter, we reported an updated meta-analysis comparing the genetic variants between PCV and nAMD. Totally 57 SNPs in 20 genes were investigated. Among them, 11 SNPs in AR*MS2-HTRA1* and rs77466370 in *FGD6* showed significant differences between PCV and nAMD, but the other SNPs had similar distribution between PCV and nAMD, including variants in *CFH, VEGF, C2, CFB*. These results suggest that PCV and nAMD shares the majority of genetic components, but the variants that distribute differently between these two conditions may explain the pathogenic and clinical difference of PCV and nAMD.

Keywords

Age-related macular degeneration
Polypoidal choroidal vasculopathy · Meta-analysis · Single nucleotide polymorphism
Genetic association

8.1 Introduction

Age-related macular degeneration (AMD) is a leading cause of blindness and central vision impairment and blindness in elderly patients [1]. There are two types of AMD, dry and wet (exudative or neovascular) AMD (nAMD), which is characterized by atrophy of retinal pigment epithelium (RPE) and choroidal neovascularization respectively. The clinical manifestations of nAMD include choroidal neovascularia-

H. Chen (✉) · X. Liao
Joint Shantou International Eye Center, Shantou University and the Chinese University of Hong Kong, Shantou, Guangdong, China

L. Ma
The First Affiliated Hospital, Dalian Medical University, Dalian, China

L. J. Chen
Department of Ophthalmology & Vision Sciences, The Chinese University of Hong Kong, Hong Kong, China

C. P. Pang
Joint Shantou International Eye Center, Shantou University and the Chinese University of Hong Kong, Shantou, Guangdong, China

Department of Ophthalmology & Vision Sciences, The Chinese University of Hong Kong, Hong Kong, China

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tion, subretinal fluid, hemorrhage, exudation, and fibrosis. Polypoidal choroidal vasculopathy (PCV) is characterized by the branching vascular network of the choroid and polyp-like aneurysmal dilations of its terminals [2]. Clinically, PCV is manifested as serosanguineous detachments of the pigmented epithelium and exudative changes that can recur in several episodes.

It is still controversy whether PCV presents a subtype of nAMD or a distinct disease. PCV and nAMD have some shared characters but there are also some different features, including risk factors, clinical manifestations, natural course, and response to treatment.

Both PCV and nAMD are commonly seen in elderly patients. However, PCV presents a younger age than nAMD [3, 4]. Although both PCV and nAMD occur in any race, PCV is known to be more prevalent in pigmented ethnicity while nAMD has a high prevalence in European than in Asian [5]. Smoking is a proven risk factors for both PCV and nAMD, while female gender is a protective factor for both conditions [3, 6]. Diabetes was found to be more prevalent in nAMD than in PCV patients [4].

Clinically, Both PCV and nAMD present as exudation or hemorrhage at the macular region. But there are also different characters between them. nAMD is predominantly located at the fovea or parafoveal region, while PCV may involve perifoveal, peripapillary, or even peripheral retina. The histological feature of PCV is majorly polypoidal enlargement of the terminal of the choroidal vessel. nAMD is characterized by choroidal neovascularization above or underneath the RPE. The choroidal thickness of nAMD is usually thin but that of PCV is usually thick. The natural history of PCV is multiple, recurrent episodes while nAMD is a progressive disease. Although both disorders can be treated using photodynamic therapy or anti-vascular endothelial growth factor (VEGF) antibody, nAMD responses better to anti-VEGF therapy and PCV responses better to photodynamic therapy [7].

Genetic studies of AMD have identified susceptibility single-nucleotide polymorphisms (SNPs) in multiple genes, including rs1061170 in *complement factor H (CFH)*, rs10490924 in *age-related maculopathy susceptibility* 2 (*ARMS2*), and rs11200638 in *high-temperature requirement factor H* (*HTRA1*) [8, 9]. In 2016, the International AMD Genomics Consortium reported 34 loci associated with AMD [10]. Due to the similarities between nAMD and PCV, major gene SNPs for nAMD have also been evaluated in PCV. The *CFH* SNP rs1061170 was not found to be associated with PCV [11], while an adjacent SNP rs800292 was significantly associated [11–13]. Both rs10490924 and rs11200638 at the *ARMS2-HTRA1* locus were associated with PCV [11, 12, 14, 15]. In 2012, we published a meta-analysis investigating genetic associations of PCV with SNPs in the *ARMS2*, *HTRA1*, *CFH*, and *complement component 2* (*C2*) genes. The results also showed that one SNP, rs10490924, in *ARMS2* showed a significant difference between PCV and AMD [16]. In 2015, we reported the updated meta-analysis of the association of genetic variants with PCV, which found 31 polymorphisms in 10 genes/loci (including ARMS2, HTRA1, CFH, C2, CFB, RDBP, SKIV2L, CETP, 8p21, and 4q12) were significantly associated with PCV. Twelve polymorphisms at the ARMS2-HTRA1 locus showed significant differences between PCV and nAMD. There are many new articles investigating these topics since the publication of the latest meta-analysis. In this chapter, we further updated our meta-analysis comparing the genetic association profiles between PCV and nAMD.

8.2 Methods of Meta-Analysis

A systematic literature search was performed using EMBASE, PubMed, Web of Science, and Chinese Biomedical Literature Database. The search used the terms (polypoidal choroidal vasculopathy or PCV) and (gene or genetic or polymorphism or variant or SNP or DNA). We retrieved all related records published from February 1, 2015, and September 27, 2018, and then added the articles published before Feb 2015 that were included in our previous meta-analysis. The reference lists of all eligible studies, reviews, and meta-analyses were also screened to prevent that any relevant studies were omitted.

The retried records were reviewed by two independent reviewers (L.M. and X. L.) and any inconsistency was resolved by discussion with another reviewer (H.C.). The following criteria were used when assessing the records [1]. case-control studies, cohort studies, or population-based studies that evaluated the difference of gene variants between PCV and nAMD; and [2] allele or genotype counts and/or frequencies being presented or able to be calculated from the data in the study. For those reports published by the same study group on the same gene markers, only the latest study was included. Case reports, animal studies, reviews, conference abstracts, comments, articles without sufficient data, or published in language other than English were excluded.

The data from included studies were extracted by the two independent reviewers (L.M. and Z.L.) and any inconsistency was resolved by discussion with another reviewer (H.C.). If there were several cohorts in the same article, they were treated as independent study. The following information from each record was extracted: first author, year of publication, the ethnicity of study subjects, study design, genotyping method, and sample size, demographics, allele, and genotype distribution in PCV and nAMD.

The distribution of genetic variants between PCV and nAMD from all included studies were pooled. Three genetic models were used, including allelic, dominant, and recessive models. The effect size was assessed using a summary odds ratio (OR) and its 95% confidence intervals (CIs) of each SNP. The software, Review Manager software (RevMan, version 5.3.5, The Cochrane Collaboration, Copenhagen, Denmark) was used for statistical analysis. The I^2 statistic was adopted to assess the heterogeneity among the studies. The I^2 values correspond with no (<25%), low (25%–50%), moderate (50%–75%), and high heterogeneity (≥75%). If the I^2 value was ≥50%, the fixed effects model was used in the meta-analysis, otherwise, the random effects model was used. A summary P value <0.05 was considered statistically significant. We performed a sensitivity analysis by omitting one study at a time and calculating the pooled ORs for the remaining studies. Funnel plots were constructed to assess potential publication bias.

8.3 Results of Updated Meta-Analysis

Our literature search yielded a total of 1315 reports published between February 1, 2015, and September 27, 2018, from EMBASE, PubMed, Web of Science and Chinese Biomedical Literature Database. Out of these, 502 articles were excluded due to duplicates. After assessing the titles and abstracts, a further 606 reports with unrelated topics were omitted. For the remaining 107 studies, the full-texts were retrieved and reviewed. Another 89 reports were excluded, among which 62 studies were on AMD but not PCV, 2 were reviews, 23 were non-genetic studies, and 1 was a case report. Finally, 18 articles were eligible for the meta-analysis. A further 66 studies published before 2015 that were used in our previous meta-analysis were added. However, 19 of these studies were excluded because they only studied in PCV patients. Thus, a total of 65 studies were included in the meta-analysis. Figure 8.1 shows the flowchart of literature inclusion and exclusion with the specification of reasons and Table 8.1 shows the characters of the included studies.

In these 65 studies, both PCV and nAMD were assessed for associations with a total of 57 SNPs in 20 genes or loci (i.e., *ARMS2*, *HTRA1*, *CFH*, *VEGF-A*, *C2*, *CFB*, *SKIV2L*, *CETP*, 8p21, 4q12, *ELN*, *LIPC*, *LPL*, *FGD6*, *ABCA1*, *ABCG1*, *PGF*, *TLR3*, *LOXL1*, and *PEDF*; Table 8.1). In total, 11 SNPs at the *ARMS2-HTRA1* locus and 1 in *FGD6* showed significant differences between PCV and nAMD (Tables 8.2 and 8.3). There was no significant difference between PCV and nAMD in the remaining 45 SNPs (Table 8.4).

There are 12 studies tested the most-investigated SNP, *ARMS2* rs10490924, involving 2361 PCV and 2138 nAMD patients (Table 8.2) [3, 12, 15, 22, 26, 27, 35, 36, 38, 43, 44, 78]. The frequency of the T allele was significantly lower in PCV than in nAMD (summary OR 0.69; 95% CI 0.63–0.75; $P = 5.50 \times 10^{-16}$;

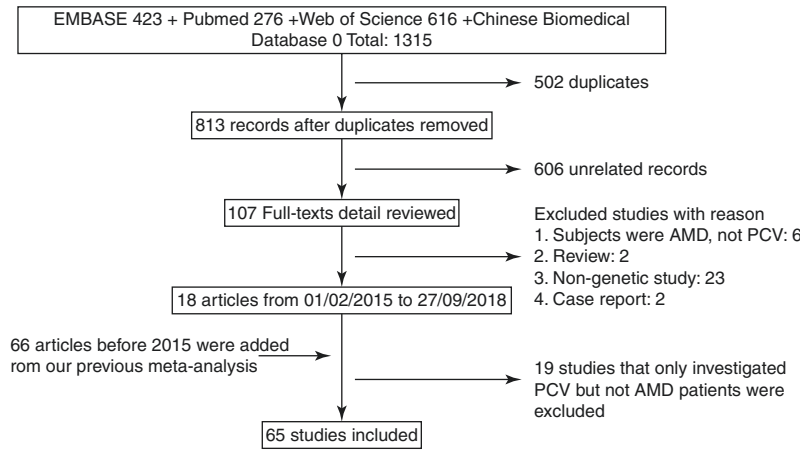

Fig. 8.1 Flow chart of literature screen

Table 8.2 and Fig. 8.2). The association was also statistically significant in both dominant and recessive models (OR = 0.64, $P = 8.80 \times 10^{-8}$ and OR = 0.62, $P = 1.47 \times 10^{-13}$ respectively; Table 8.3 and Fig. 8.2). The results of the sensitivity analysis found that the association remains significant after omitting any single included cohorts (data not shown). And there was no asymmetry on the funnel plots (Fig. 8.5). There are 8 other SNPs in *ARMS2*, namely rs3750848, rs36212731, rs36212732, rs36212733, rs3750846, rs10664316, c.372_815del443ins54 and rs2672587, were evaluated in 2 to 3 cohorts, and also showed significant differences between PCV and nAMD (ORs values between 0.48 and 0.71, P values between 7.19×10^{-9} and 0.05; Table 8.2).

There are seven studies tested the *HTRA1* SNP rs11200638 in 1362 PCV and 1364 nAMD patients [3, 14, 18, 43, 44, 57, 73]. The A allele frequency was lower in PCV compared to nAMD, with a summary OR of 0.75 (95% CI, 0.67–0.84; $P = 2.14 \times 10^{-5}$; Table 8.2 and Fig. 8.3). The association was also statistically significant in both dominant and recessive models (OR = 0.67, P = 0.006 and OR = 0.70, $P = 9.87 \times 10^{-6}$ respectively, Table 8.3 and Fig. 8.3). The results of the sensitivity analysis found that the association remain significant after omitting any single included cohorts (data not shown). And there was no asymmetry on the funnel plots (Fig. 8.5). Another *HTRA1* SNP, rs2672587, was also evaluated in two cohorts, and showed significant differences between PCV and nAMD (G allele; OR, 1.41; 95% CI, 1.07–1.85; $P = 0.01$; Table 8.2).

The SNP rs77466370 in *FGD6* was studied in 3318 PCV and 2457 nAMD patients from five cohorts. The summary OR for the T allele was 1.86 (95% CI, 1.48–2.35; $P = 1.29 \times 10^{-7}$; Table 8.2 and Fig. 8.4). The association was statistically significant in the dominant model but no in the recessive model (OR = 1.89, $P = 1.52 \times 10^{-7}$ and OR = 2.19, $P = 0.27$ respectively; Table 8.3 and Fig. 8.4). The results of the sensitivity analysis found that the association remain significant after omitting any single included cohorts (data not shown). And there was no asymmetry on the funnel plots (Fig. 8.5).

8.4 Discussion

Genetic variants are important risk factors for both nAMD and PCV. This updated systematic review and meta-analysis compared the distribution of genetic variants between nAMD and PCV. The results showed that 57 SNPs in 20 genes had been investigated in both PCV and nAMD in the same cohorts. The pooled outcomes showed 11 SNPs at the *ARMS2-HTRA1* locus and 1 SNP in *FGD6* had significant differences between PCV and nAMD. The results are robust because the sensitivity test found consistency when omitting any included studies. There was no publication bias found on the funnel plots. There was no significant difference between PCV and nAMD in

Table 8.1 Characteristics of the included studies in the meta-analysis

First author and reference	Year	Ethnicity	Study design	Genotyping method	HWE reported	PCV			AMD			Gene/loci investigated
						N	Male ratio	Mean age ± SD (years)	*N*	Male ratio	Mean age ± SD (years)	
						N	Male ratio	Mean age ± SD (years)	N	Male ratio	Mean age ± SD (years)	
Gotoh N [17]	2004	Japanese	1	PCR	Yes	58	0.76	71.6 ± 6.2	85	0.81	70.9 ± 7.9	*APOE*
Kondo N[a] [18]	2007	Japanese	1	TaqMan	Yes	76	0.82	73.6 ± 7.4	73	0.79	75.7 ± 7.3	*ARMS2*, *HTRA1*
Gotoh N[a] [19]	2008	Japanese	1	TaqMan	NA	204	0.72	73.1 ± 7.7	116	0.74	76.1 ± 8.3	*CFH*, *HTRA1*
Kondo N[a] [20]	2008	Japanese	2	TaqMan	Yes	103	0.81	74 ± 6.6	78	0.78	76 ± 7.4	*ELN*
Bessho H[a] [21]	2009	Japanese	2	TaqMan	Yes	140	0.77	73 ± 6.9	116	0.78	75 ± 7.2	*PEDF*
Goto A[a] [22]	2009	Japanese	2	TaqMan	Yes	100	0.81	72.7 ± 8.3	100	0.73	74.6 ± 8.8	*ARMS2*, *CFH*, *C3*
Gotoh N[a] [23]	2009	Japanese	2	PCR	Yes	55	0.82	72.9 ± 7.3	56	0.64	76.2 ± 9.1	*ARMS2*
Kondo N [24]	2009 a	Japanese	2	TaqMan	Yes	140	0.77	73 ± 6.9	116	0.78	75 ± 7.2	*SOD2*
Gotoh N[a] [14]	2010	Japanese	2	TaqMan	Yes	181	0.72	73.0 ± 7.83	84	0.74	76.2 ± 8.58	*ARMS2*, *HTRA1*
Hayashi H[a] [25]	2010	Japanese	2	TaqMan	Yes	518	0.73	75.0 ± 7.8	408	0.72	77.4 ± 8.4	*ARMS2*, *CFH*
Lima LH[a] [15]	2010	Caucasian	2	PCR & TaqMan	NA	55	0.58	73 ± 8.2	368	–	–	*ARMS2*, *CFH*, *CFB*, *C2*
Bessho H[a] [26]	2011	Japanese	3	TaqMan	NA	119	0.81	73 ± 8.0	68	0.78	76 ± 7.0	*ARMS2*
Fuse N[a] [27]	2011	Japanese	2	PCR	Yes	60	0.80	70.3 ± 9.2	50	0.80	71.3 ± 8.2	*ARMS2*, *LOXL1*
Lima LH[a] [28]	2011	Caucasian	2	TaqMan	NA	56	0.59	72 ± 8.3	368	–	71.3 ± 8.9	*ELN*
Nakata I [29]	2011 b	Japanese	2	TaqMan	Yes	510	0.73	77.4 ± 8.4	401	0.72	75.0 ± 7.8	*SERPING1*
Sng CCA[a] [30]	2011	Chinese	1	PCR	Yes	120	0.64	68.8 ± 7.9	126	0.67	73.0 ± 8.6	*TLR3*
Yamashiro K[a] [31]	2011	Japanese (Kyoto)	2	TaqMan	NA	518	0.74	75.1 ± 8.5	408	0.72	77.7 ± 8.4	*ELN*
		Japanese (Saitama)	2	TaqMan	NA	154	0.79	71.8 ± 7.8	216	0.73	72.7 ± 8.7	*ELN*
Zhang X [32]	2011	Chinese	2	PCR	Yes	177	0.66	65 ± 8.45	131	0.64	67 ± 9.46	9q21

(continued)

Table 8.1 (continued)

First author and reference	Year	Ethnicity	Study design	Genotyping method	HWE reported	PCV			AMD			Gene/loci investigated
						N	Male ratio	Mean age ± SD (years)	*N*	Male ratio	Mean age ± SD (years)	
Wu K[a] [33]	2012	Chinese	2	PCR	Yes	177	0.66	65 ± 8.45	131	0.64	67 ± 9.46	*PEDF*
Arakawa S[a] [10]	2011	Japanese	2	BeadChip & PCR	NA	480	–	–	298	–	–	8p21, 4q12
Sakurada Y[a] [34]	2011	Japanese	2	PCR	Yes	135	–	–	89	–	–	*LOXL1*
Tanaka K[a] [35]	2011	Japanese	2	TaqMan	Yes	381	0.71	69.9 ± 9.1	253	0.74	73.7 ± 7.5	*CFH*, *ARMS2*, *ELN*, *MTHFR*
Yanagisawa S[a] [36]	2011	Japanese	2	TaqMan	Yes	198	0.79	73 ± 7.3	181	0.77	75 ± 7.4	*ARMS2*
Bessho H [37]	2012	Japanese	2	TaqMan	Yes	210	0.79	73.8 ± 7.5	139	0.78	75.3 ± 7.3	*CD36*
Liang XY[a] [38]	2012	Chinese	2	PCR	Yes	164	0.69	67.5 ± 9.0	156	0.53	75.9 ± 7.4	*ARMS2*, *HTRA1*
Nakata I[a] [39]	2012 a	Japanese (Kyoto)	2	TaqMan & Beadchip	Yes	720	–	–	664	–	–	8p21, 4q12
		Japanese (Yamanashi)	2	TaqMan	Yes	211	–	–	112	–	–	8p21, 4q12
		Chinese (Singapore)	2	Beadchip	Yes	118	–	–	122	–	–	8p21, 4q12
		Chinese (Hong Kong)	2	TaqMan	Yes	200	–	–	233	–	–	8p21, 4q12
Nakata I[a] [40]	2012 b	Japanese	2	TaqMan	Yes	581	0.72	72.59 ± 8.13	455	0.73	75.59 ± 8.60	*C2/CFB*
Nishiguchi KM [41]	2012	Japanese	2	PCR	Yes	105	0.85	70.2 ± 6.7	198	0.72	73.1 ± 8.0	*C9*
Zeng R [42]	2012	Chinese	2	PCR	Yes	195	0.67	64 ± 8.75	136	0.63	67 ± 9.29	*TIMP3*
Zuo C [43]	2012	Chinese	2	PCR	Yes	195	0.67	64 ± 8.75	136	0.63	67 ± 9.29	*COL1A2*
Cheng Y[a] [44]	2013	Chinese	2	PCR	Yes	92	0.52	69.5 ± 9.4	96	0.65	70.3 ± 8.8	*ARMS2*, *HTRA1*
Guo J [45]	2013	Chinese	2	PCR	Yes	300	0.63	66.8 ± 9.7	300	0.63	69.4 ± 8.9	*TOMM40*
Liu K[a] [46]	2013	Chinese	2	TaqMan	Yes	233	0.70	68.5 ± 9.0	200	0.55	75.3 ± 7.7	*C2-CFB-RDBP-SKIV2L*, *CFH*, *HTRA1*
Su Y [47]	2013	Chinese	2	PCR	Yes	251	0.67	65 ± 8.61	157	0.64	67 ± 9.21	*VEGF-A*

Sun Y[a] [48]	2013	Chinese	2	PCR	Yes	300	0.63	66.8 ± 9.7	300	0.63	69.4 ± 8.9	8p21, 4q12
Zhang X[a] [49]	2013	Chinese	2	PCR	Yes	250	0.66	65 ± 8.60	157	0.64	67 ± 9.21	*LIPC*, *ABCA1*, *CETP*, *LPL*, *FADS1*, *CFH*, *ARMS2*, and near *HTRA1*
Cheng Y[a] [50]	2014	Chinese	2	PCR	NA	92	0.52	69.5 ± 9.4	96	0.65	70.3 ± 8.8	*TLR3*
Huang L[a] [51]	2014a	Chinese	2	PCR	Yes	368	0.61	66.6 ± 9.6	344	0.64	69.2 ± 8.7	*CFH*
Huang L[a] [52]	2014b	Chinese	2	PCR	Yes	300	0.63	66.8 ± 9.6	300	0.63	69.4 ± 8.9	*FEK/COL10A1*, *VEGF-A*
Hata M [53]	2014	Japanese	3	TaqMan	NA	70	0.81	72.2 ± 8.8	58	0.71	76.4 ± 8.2	*CFH*, *ARMS2*
Ji Y[a] [54]	2014	Chinese	2	PCR	Yes	251	0.67	65 ± 8.6	157	0.64	67 ± 9.2	*GDF6*
Li F[a] [55]	2014	Chinese	2	PCR	Yes	298	0.62	66.8 ± 9.7	300	0.63	69.4 ± 8.9	*ABCA1*
Liang XY[a] [56]	2014	Chinese	2	PCR	Yes	179	0.71	67.9 ± 9.0	155	0.55	75.6 ± 7.5	*FPR1*
Liu K[a] [57]	2014a	Chinese	2	TaqMan	Yes	233	0.70	68.5 ± 9.0	200	0.55	75.3 ± 7.7	*ABCA1*, *LIPC*, *CETP*, *LCAT*, *PLTP*, *ABCG1*, *CFH*, *HTRA1*
Liu K[a] [58]	2014b	Chinese	2	TaqMan	Yes	233	0.70	68.5 ± 9.0	200	0.55	75.3 ± 7.7	*C3*, *CFH*, *HTRA1*
Park DH[a] [59]	2014	Korean	2	PCR	NA	62	0.79	70.3 ± 6.9	42	0.71	70.9 ± 7.2	*ARMS2*
Tanaka K[a] [60]	2014	Japanese	2	TaqMan	Yes	376	0.71	70.0 ± 8.9	250	0.75	73.6 ± 7.5	*C2*, *CFB*
Yang F [61]	2014	Chinese	2	PCR	Yes	300	0.63	66.8 ± 9.6	300	0.63	69.4 ± 8.9	*CFI*
Yoneyama S[a] [62]	2014 a	Japanese	1	TaqMan	Yes	250	0.76	73.1 ± 8.2	132	0.72	76.9 ± 7.7	*CFH*, *ARMS2*
Yoneyama S[a] [63]	2014 b	Japanese	1	TaqMan	Yes	333	0.76	73.1 ± 8.2	157	0.73	75.6 ± 8.4	*CFH*, *ARMS2*, *SKIV2L*
Zeng R [64]	2014	Chinese	2	PCR	Yes	251	0.67	66 ± 9.44	157	0.64	67 ± 9.17	*MMP9*
Huang L [65]	2015	Chinese	2	MALDI-TOF-MS	Yes	300	0.63	66.8 ± 9.7	300	63%	69.4 ± 8.9	*CFH, ARMS2/HTRA1*
Chen LJ [66]	2015	Chinese	2	TaqMan	Yes	236	0.69	68.5 ± 9.0	214	0.57	75.2 ± 7.6	*PGF, VEGF-A, VEGFB*
Jin E [67]	2015	Chinese	2	PCR	Yes	174	0.54	64.52 ± 6.78	453	0.56	67.12 ± 6.81	*HERPUD1*
Meng Q [68]	2015	Chinese	2	MALDI-TOF-MS	Yes	291	0.60	66.6 ± 9.6	230	0.63	69.3 ± 8.8	*CETP, LIPC*
Woo S [3]	2015	Korean	2	PCR	Yes	111	0.55	67.35 ± 7.34	154	0.56	72.56 ± 8.10	*31 candidate genes*
Yu Y [69]	2015	Chinese	2	MALDI-TOF-MS	Yes	300	0.63	66.8 ± 9.7	300	0.63	69.4 ± 8.9	*COL8A1*
Huang L [70]	2016	Chinese, Japanese, and Korean	2	Exome sequencing	Yes	3318	–	–	2457	–	–	

(continued)

Table 8.1 (continued)

First author and reference	Year	Ethnicity	Study design	Genotyping method	HWE reported	PCV			AMD			Gene/loci investigated
						N	Male ratio	Mean age ± SD (years)	*N*	Male ratio	Mean age ± SD (years)	
Ma L [71]	2016	Hong Kong Chinese	2	TaqMan	Yes	236	0.69	68.5 ± 9.0	235	0.55	75.3 ± 7.6	*ABCG1*
		Shantou Chinese	2	TaqMan	Yes	187	0.72	63.1 ± 10.5	189	0.69	67.3 ± 10.1	*ABCG1*
		Japanese	2	TaqMan	Yes	204	0.77	72.2 ± 8.0	192	0.67	74.3 ± 7.3	*ABCG1*
Ng TK [72]	2016	Chinese	2	PCR	Yes	188	–	–	195	–	–	*HTRA1*
Ye Z [73]	2016	Chinese	2	SNaPshot	Yes	419	0.70	64.8 ± 9.7	490	0.62	67.5 ± 9.6	*6p21.3, CFH, HTRA1*
Zuo C [74]	2016	Chinese	2	SNaPshot	Yes	250	0.66	65 ± 8.60	157	0.64	67 ± 9.21	*ENOS*
Fan Q [75]	2017	East Asian	2	Beadchips	Yes	1157	–	–	1062	–	–	*34 known AMD loci*
Ma L [76]	2017	Hong Kong Chinese	2	TaqMan	Yes	236	0.69	68.5 ± 9.0	214	0.57	75.2 ± 7.6	*ANGPT2, CFH*
		Shantou Chinese	2	TaqMan	Yes	187	0.72	63.1 ± 10.5	189	0.69	67.3 ± 10.1	*ANGPT2, CFH*
		Japanese	2	TaqMan	Yes	204	0.77	72.2 ± 8.0	192	0.67	74.3 ± 7.3	*ANGPT2, CFH*
Wen X [77]	2018	Chinese		Whole-exome and Sequenom MassARRAY	Yes	180	–	–	166	–	–	

Study design: 1 = cross-sectional study; 2 = case-control study; 3 = cohort study

[a]Indicates the study included both PCV and nAMD; "-" indicates there was no data for the ascertainment of controls; *NA* not mentioned in original studies

nAMD neovascular age-related macular degeneration; *PCR* polymerase chain reaction; *PCV* polypoidal choroidal vasculopathy

Table 8.2 Polymorphisms with significant differences between PCV and neovascular AMD

Region	Gene	Polymorphism	Ethnicity	Associated vs. reference allele	No. of cohorts	PCV vs. nAMD (sample size)	OR (95% CI)	*P*	I^2 (%)
10q26	*ARMS2*	rs10490924	All ancestries	T vs. G	12	2361 vs. 2138	0.69 (0.63–0.75)	5.50E−16	29
10q26	*ARMS2*	rs3750848	Asian	G vs. T	3	316 vs. 304	0.60 (0.47–0.76)	2.14E−05	0
10q26	*ARMS2*	rs36212731	Asian	T vs. G	3	1279 vs. 1364	0.71 (0.63–0.79)	2.23E−09	48
10q26	*ARMS2*	rs36212732	Asian	G vs. A	2	217 vs. 207	0.57 (0.42–0.76)	0.0001	0
10q26	*ARMS2*	rs36212733	Asian	C vs. T	2	217 vs. 204	0.57 (0.42–0.76)	0.0001	0
10q26	*ARMS2*	rs3750846	Asian	C vs. T	2	216 vs. 204	0.57 (0.42–0.76)	0.0001	0
10q26	*ARMS2*	rs10664316	Asian	I vs. N[a]	2	218 vs. 208	0.48 (0.33–0.69)	8.49E−05	45
10q26	*ARMS2*	c.372_815del443ins54	Asian	D vs. N†	2	219 vs. 209	0.55 (0.41–0.73)	4.50E−05	0
10q26	*ARMS2*	rs2014307	Asian	G vs. T	2	216 vs. 212	0.52 (0.27–1.00)	0.05	64
10q26	*HTRA1*	rs11200638	Asian	A vs. G	7	1362 vs. 1251	0.75 (0.67–0.84)	8.65E−07	32
10q26	*HTRA1*	rs2672587	Asian	G vs. C	2	276 vs. 182	1.41 (1.07–1.85)	0.01	9
12q22	FGD6	rs77466370	Asian	T vs. C	5	3318 vs. 2457	1.86 (1.48–2.35)	1.29E−07	0

Gene symbols: *ARMS2 = age-related maculopathy susceptibility 2*; *HTRA1 = HtrA serine peptidase 1*
[a]insAT vs. wide type; † del443ins54 vs. wide type
AMD age-related macular degeneration; *CI* confidence interval; *OR* odds ratio; *PCV* polypoidal choroidal vasculopathy; *Ref* reference

Table 8.3 Gene SNPs with significant differences between PCV and nAMD in dominant, recessive, homozygous and heterozygous models

Region	Gene	Polymorphism	Ethnicity	Associated vs. reference genotype	No. of cohorts	PCV vs. nAMD (sample size)	OR (95% CI)	*P*	I^2 (%)
10q26	*ARMS2*	rs10490924	All ancestries	TT + TG vs. GG	12	2361 vs. 2138	0.64 (0.54–0.75)	8.80E−08	0
				TT vs. TG + GG			0.62 (0.55–0.71)	1.47E−13	49
10q26	*ARMS2*	rs3750848	Asian	GG + GT vs. TT	3	316 vs. 304	0.78 (0.50–1.22)	0.28	0
				GG vs. GT + TT			0.44 (0.32–0.61)	6.70E−07	0
10q26	*ARMS2*	rs36212731	Asian	TT + TG vs. GG	2	1279vs. 1364	0.64 (0.55–0.75)	0.0001	0
				TT vs. TG + GG			0.64 (0.55–0.75)	2.70E−08	67
10q26	*ARMS2*	rs36212732	Asian	GG + GA vs. AA	2	219 vs. 212	0.84 (0.50–1.41)	0.51	0
				GG vs. GA + AA			0.47 (0.32–0.70)	0.0002	0
10q26	*ARMS2*	rs36212733	Asian	CC + CT vs. TT	2	217 vs. 204	0.84 (0.50–1.41)	0.51	0
				CC vs. CT + TT			0.47 (0.32–0.70)	0.0002	16
10q26	*ARMS2*	rs3750846	Asian	CC + CT vs. TT	2	216 vs. 204	0.65 (0.37–1.15)	0.14	0
				CC vs. CT + TT			0.44 (0.30–0.66)	4.70E−05	0
10q26	*ARMS2*	rs10664316	Asian	II + IN vs. NN[a]	2	218 vs. 208	0.35 (0.12–0.99)	0.05	0
				II vs. IN+NN[a]			0.50 (0.24–1.00)	0.05	57
10q26	*ARMS2*	c.372_815del443ins54	Asian	DD + DN vs. NN†	2	219 vs. 209	0.56 (0.32–0.98)	0.04	0
				DD vs. DN + NN†			0.45 (0.31–0.66)	5.58E−05	0
10q26	*ARMS2*	rs2014307	Asian	GG + GT vs. TT	2	216 vs. 212	0.37 (0.13–1.06)	0.06	0
				GG vs. GT + TT			0.49 (0.22–1.13)	0.09	68
10q26	*HTRA1*	rs11200638	Asian	AA+AG vs. GG	7	1362 vs. 1251	0.67 (0.53–0.84)	0.0006	13
				AA vs. AG + GG			0.70 (0.60–0.82)	9.87E−06	35
10q26	*HTRA1*	rs2672587	Asian	GG + GC vs. CC	2	276 vs. 182	1.74 (0.89–3.43)	0.11	64
				GG vs. GC + CC			1.32 (0.80–2.18)	0.28	0
12q22	FGD6	rs77466370	Asian	TT + TC vs. CC	5	3318 vs. 2457	1.89 (1.49–2.40)	1.52E−07	0
				TT vs. TC + CC			NA	NA	NA

Gene symbols: *ARMS2* age-related maculopathy susceptibility 2; *HTRA1* HtrA serine peptidase 1

[a]I vs. N = insAT vs. wide type; † D vs. N = del443ins54 vs. wide type

CI confidence interval; *nAMD* neovascular AMD; *OR* odds ratio; *PCV* polypoidal choroidal vasculopathy

Table 8.4 Gene variants not significantly different between PCV and neovascular AMD

Region	Gene	Polymorphism	Ethnicity	Associated vs. reference genotype	No. of cohorts	PCV vs. nAMD (sample size)	OR (95% CI)	*P*	I^2 (%)
1q32	*CFH*	rs800292	Asian	A vs. G	12	3344 vs. 2803	0.98 (0.90–1.06)	0.57	24
				AA+AG vs. GG			0.99 (0.90–1.10)	0.91	9
				AA vs. AG + GG			1.01 (0.84–1.23)	0.88	0
1q32	*CFH*	rs1061170	All ancestries	C vs. T	3	1127 vs. 1268	0.86 (0.72–1.03)	0.11	0
				CC + CT vs. TT			0.87 (0.71–1.08)	0.20	0
				CC vs. CT + TT			0.71 (0.42–1.19)	0.19	0
1q32	*CFH*	rs1410996	All ancestries	T vs. C	3	617 vs. 867	0.99 (0.73–1.80)	0.94	64
				TT + TC vs. CC			0.96 (0.68–1.34)	0.81	49
				TT vs. TC + CC			0.89 (0.61–1.31)	0.56	6
1q32	*CFH*	rs529825	All ancestries	A vs. G	2	423 vs. 712	0.98 (0.48–2.00)	0.96	85
				AA+AG vs. GG			0.98 (0.43–2.22)	0.96	84
				AA vs. AG + GG			0.66 (0.38–1.13)	0.13	35
1q32	*CFH*	rs3766404	Asian	C vs. T	2	249 vs. 523	1.14 (0.74–1.75)	0.55	0
				CC + CT vs. TT			1.10 (0.70–1.75)	0.68	0
				CC vs. CT + TT			1.39 (0.22–8.64)	0.72	0
4q12		rs1713985	Asian	G vs. T	6	2062 vs. 1611	0.99 (0.90–1.10)	0.86	0
				GG + GT vs. TT			0.99 (0.87–1.13)	0.93	0
				GG vs. GT + TT			0.97 (0.77–1.22)	0.79	0
4q35.1	TLR3	rs3775291	Chinese	T vs. C	2	201 vs. 172	1.25 (0.92–1.70)	0.15	0
				TT + TC vs. CC			1.47 (0.95–2.26)	0.08	0
				TT vs. TC + CC			1.15 (0.61–2.17)	0.66	0
6p21	*C2*	rs547154	Asian	T vs. G	5	1439 vs. 1428	1.08 (0.84–1.40)	0.55	16
				TT + TG vs. GG			1.09 (0.83–1.42)	0.54	19
				TT vs. TG + GG			1.16 (0.29–4.62)	0.83	0
6p21	*CFB*	rs4151667	All ancestries	A vs. T	4	858 vs. 973	1.19 (0.70–2.04)	0.53	0

(continued)

Table 8.4 (continued)

Region	Gene	Polymorphism	Ethnicity	Associated vs. reference genotype	No. of cohorts	PCV vs. nAMD (sample size)	OR (95% CI)	*P*	I^2 (%)
				AA+AT vs. TT			1.11 (0.63–1.96)	0.72	0
				AA vs. AT+TT			NA	NA	NA
6p21	*CFB*	rs2072633	Asian	G vs. A	3	1190 vs. 905	1.02 (0.90–1.15)	0.75	22
				GG + GA vs. AA			1.01 (0.82–1.23)	0.94	0
				GG vs. GA + AA			1.04 (0.85–1.27)	0.71	0
6p21	*SKIV2L*	rs429608	Asian	A vs. G	3	985 vs. 847	1.77 (1.12–2.81)	0.01	47
				AA+AG vs. GG			1.79 (1.12–2.85)	0.02	45
				AA vs. AG + GG			NA	NA	NA
6p21	*SKIV2L*	rs401775	Chinese	C vs. T	2	1295 vs. 1357	1.01 (0.84–1.22)	0.90	0
				CC + CT vs. TT			1.02 (0.83–1.24)	0.88	0
				CC vs. CT + TT			1.06 (0.49–2.30)	0.88	0
6p21.1	*VEGF-A*	rs833069	Asian	G vs. T	2	362 vs. 327	1.61 (0.82–3.17)	0.17	88
				GG + GT vs. TT			1.67 (0.81–3.44)	0.16	79
				GG vs. GT + TT			2.22 (0.64–7.76)	0.21	84
6p21.1	*VEGF-A*	rs833069	Asian	G vs. T	3	681 vs. 526	1.05 (0.89–1.24)	0.59	28
				GG + GT vs. TT			1.00 (0.79–1.26)	0.98	33
				GG vs. GT + TT			1.19 (0.87–1.63)	0.28	0
6p21.1	*VEGF-A*	rs943080	Asian	C vs. T	2	487 vs. 371	0.87 (0.69–1.10)	0.25	0
				CC + CT vs. TT			0.85 (0.64–1.12)	0.24	41
				CC vs. CT + TT			0.91 (0.47–1.73)	0.76	0
7q11	*ELN*	rs884843	Asian	G vs. A	3	760 vs. 665	0.94 (0.81–1.09)	0.41	43
				GG + GA vs. AA			0.93 (0.74–1.16)	0.52	31
				GG vs. GA + AA			0.91 (0.70–1.19)	0.49	15
7q11	*ELN*	rs13239907	Asian	G vs. A	3	766 vs. 683	0.97 (0.83–1.13)	0.70	0

Table 8.4 (continued)

Region	Gene	Polymorphism	Ethnicity	Associated vs. reference genotype	No. of cohorts	PCV vs. nAMD (sample size)	OR (95% CI)	*P*	I^2 (%)
				GG + GA vs. AA			0.91 (0.66–1.25)	0.56	0
				GG vs. GA + AA			0.99 (0.80–1.22)	0.89	0
7q11	*ELN*	rs2856728	Asian	C vs. T	3	758 vs. 670	1.05 (0.63–1.75) 0.90 (0.76–1.06)	0.84 0.22	85
				CC + CT vs. TT			1.09 (0.64–1.87)	0.75	80
				CC vs. CT + TT			0.83 (0.34–2.03)	0.68	69
7q11	*ELN*	rs868005	Asian	C vs. T	3	650 vs. 539	1.06 (0.87–1.29)	0.54	26
				CC + CT vs. TT			1.13 (0.89–1.42)	0.32	16
				CT vs. TT			1.15 (0.79–1.67)	0.46	32
7q11	*ELN*	rs2301995	All ancestries	A vs. G	5	1203 vs. 1305	1.09 (0.74–1.59)	0.67	80
				AA+AG vs. GG			1.14 (0.70–1.86)	0.59	80
				AA vs. AG + GG			0.77 (0.53–1.12)	0.17	14
8p21.3	LPL	rs12678919	Asian	G vs. A	2	827 vs. 997	0.99 (0.81–1.20)	0.89	0
				GG + GA vs. AA			1.02 (0.82–1.27)	0.83	0
				GG vs. GA + AA			0.54 (0.23–1.31)	0.17	28
				CC vs. CT + TT			3.42 (0.59–19.64)	0.17	0
8p21.3	*LPL*	rs12678919	Asian	G vs. A	2	541 vs. 387	1.16 (0.86–1.57)	0.32	0
				GG + GA vs. AA			1.18 (0.85–1.64)	0.32	0
				GG vs. GA + AA			1.20 (0.35–4.17)	0.77	0
8p21		rs13278062	Asian	T vs. G	6	2062 vs. 1611	1.00 (0.91–1.10)	0.96	0
				TT + TG vs. GG			1.01 (0.88–1.15)	0.94	0
				TT vs. TG + GG			1.01 (0.83–1.22)	0.94	0
				AA vs. AC + CC			1.86 (0.65–5.37)	0.25	43
8p23.1	*ANGPT2*	rs2515487	Asian	A vs. C	3	627 vs. 595	1.05 (0.88–1.25)	0.62	18
				AA+AC vs. CC			1.05 (0.84–1.32)	0.66	0

(continued)

Table 8.4 (continued)

Region	Gene	Polymorphism	Ethnicity	Associated vs. reference genotype	No. of cohorts	PCV vs. nAMD (sample size)	OR (95% CI)	*P*	I^2 (%)
				AA vs. AC + CC			1.11 (0.73–1.68)	0.63	0
8p23.1	*ANGPT2*	rs2922869	Asian	T vs. C	3	627 vs. 595	1.15 (0.96–1.37)	0.12	0
				TT + TC vs. CC			1.25 (0.81–1.91))	0.31	0
				TT vs. TC + CC			1.18 (0.94–1.47)	0.16	0
8p23.1	*ANGPT2*	rs13255574	Asian	C vs. T	3	627 vs. 595	0.99 (0.80–1.22)	0.91	2
				CC + CT vs. TT			0.99 (0.51–1.90)	0.97	0
				CC vs. CT + TT			0.98 (0.77–1.25)	0.86	0
8p23.1	*ANGPT2*	rs4455855	Asian	G vs. A	3	627 vs. 595	0.93 (0.79–1.10)	0.41	30
				GG + GA vs. AA			0.90 (0.66–1.22)	0.50	0
				GG vs. GA + AA			0.92 (0.73–1.17)	0.50	0
8p23.1	*ANGPT2*	rs11775442	Asian	A vs. G	3	627 vs. 595	0.92 (0.75–1.13)	0.42	0
				AA+AG vs. GG			0.86 (0.46–1.60)	0.64	0
				AA vs. AG + GG			0.92 (0.73–1.17)	0.49	0
9q31.1	*ABCA1*	rs1883025	Chinese	T vs. C	3	779 vs. 657	0.98 (0.82–1.17)	0.82	0
				TT + TC vs. CC			0.98 (0.79–1.21)	0.84	0
				TT vs. TC + CC			0.96 (0.61–1.53)	0.88	0
				TT vs. TC + CC			2.83 (0.59–13.45)	0.19	0
10q26	*ARMS2*	rs2736912	Asian	T vs. C	2	229 vs. 212	1.17 (0.45–3.04)	0.74	78
				TT + TC vs. CC			1.10 (0.37–3.28)	0.86	80
				TT vs. TC + CC			3.42 (0.56–21.04)	0.19	0
10q26	*ARMS2*	rs3750847	Asian	T vs. C	2	519 vs. 505	0.84 (0.69–1.01)	0.06	86
				TT + TC vs. CC			0.97 (0.67–1.41)	0.88	53
				TT vs. TC + CC			0.44 (0.30–0.66)	4.91E −05	0
10q26	*HTRA1*	rs11200644	Asian	C vs. T	2	281 vs. 182	1.40 (0.94–2.09)	0.10	0
				CC + CT vs. TT			1.35 (0.87–2.11)	0.18	0

Table 8.4 (continued)

Region	Gene	Polymorphism	Ethnicity	Associated vs. reference genotype	No. of cohorts	PCV vs. nAMD (sample size)	OR (95% CI)	*P*	I^2 (%)
				CC vs. CT + TT			3.42 (0.59–19.64)	0.17	0
10q26	*HTRA1*	rs7093894	Japanese	A vs. C	2	281 vs. 184	1.41 (0.99–2.01)	0.06	0
				AA+AC vs. CC			1.43 (0.95–2.16)	0.08	0
				AA vs. AC + CC			1.86 (0.65–5.37)	0.25	43
10q26	*HTRA1*	rs3793917	Asian	C vs. G	2	481 vs. 384	1.15 (0.95–1.40)	0.16	73
				CC + CG vs. GG			1.34 (0.96–1.88)	0.08	46
				CC vs. CG + GG			1.09 (0.82–1.45)	0.56	64
11q12.1	*SERPING1*	rs2511989	Asian	A vs. G	2	628 vs. 1643	0.90 (1.75–1.09)	0.30	22
				AA+AG vs. GG			0.93 (0.75–1.15)	0.50	0
				AA vs. AG + GG			0.82 (0.12–5.46)	0.84	61
14q24.3	*PGF*	rs2268615	Asian	G vs. C	2	423 vs. 403	0.74 (0.58–0.93)	0.01	0
				GG + GC vs. CC			0.60 (0.32–1.13)	0.11	0
				GG vs. GC + CC			0.70 (0.53–0.93)	0.01	0
14q24.3	*PGF*	rs2268614	Asian	G vs. C	2	423 vs. 403	0.75 (0.59–0.95)	0.02	0
				GG + GC vs. CC			0.58 (0.30–1.11)	0.10	0
				GG vs. GC + CC			0.74 (0.56–0.98)	0.03	0
15q21.3	LIPC	rs493258	Chinese	G vs. T	2	483 vs. 357	1.12 (0.90–1.41)	0.31	0
				GG + GT vs. TT			1.15 (0.87–1.52)	0.33	0
				GG vs. GT + TT			1.23 (0.70–2.19)	0.47	0
15q21.3	LIPC	rs10468017	Chinese	T vs. C	3	804 vs. 587	1.02 (0.83–1.26)	0.83	0
				TT + TC vs. CC			1.03 (0.82–1.31)	0.79	0
				TT vs. TC + CC			0.97 (0.49–1.91)	0.92	0
15q24.1	*LOXL1*	rs1048661	Japanese	T vs. G	2	195 vs. 139	0.80 (0.58–1.09)	0.15	0
				TT + TG vs. GG			0.77 (0.45–1.32)	0.35	0
				TT vs. TG + GG			0.73 (0.46–1.16)	0.18	0

(continued)

Table 8.4 (continued)

Region	Gene	Polymorphism	Ethnicity	Associated vs. reference genotype	No. of cohorts	PCV vs. nAMD (sample size)	OR (95% CI)	*P*	I^2 (%)
16q13	CETP	rs3764261	Chinese	T vs. C	3	774 vs. 587	1.09 (0.91–1.31)	0.34	0
				TT + TC vs. CC			1.11 (0.89–1.38)	0.37	0
				TT vs. TC + CC			1.12 (0.68–1.85)	0.66	0
16q13	CETP	rs2303790	Chinese	G vs. A	2	170 vs. 143	1.21 (0.96–1.51)	0.11	80
				GG + GA vs. AA			1.20 (0.95–1.52)	0.13	78
				GG vs. GA + AA			NA	NA	NA
17p13.3	*PEDF*	rs1136278	Asian	T vs. G	2	317 vs. 247	1.07 (0.72–1.61)	0.73	65
				TT + TG vs. GG			1.29 (0.64–2.58)	0.47	70
				TT vs. TG + GG			0.91 (0.62–1.34)	0.64	0
21q22.3	*ABCG1*	rs57137919	Asian	A vs. G	3	627 vs. 616	1.16 (0.97–1.39)	0.11	0
				AA+AG vs.GG			1.18 (0.94–1.47)	0.16	0
				AA vs. AG + GG			1.29 (0.82–2.04)	0.27	0
21q22.3	*ABCG1*	rs225396	Asian	T vs. C	3	627 vs. 616	1.12 (0.95–1.32)	0.19	0
				TT + TC vs. CC			1.14 (0.91–1.44)	0.24	0
				TT vs. TC + CC			1.19 (0.86–1.65)	0.30	0

Gene symbols: *ABCA1* ATP-binding cassette-sub-family A (ABC1)-member 1; *ARMS2* age-related maculopathy susceptibility 2; C2 complement component 2; *C4orf14* nitric oxide associated 1; *CETP* cholesteryl ester transfer protein-plasma; *CFB* complement factor B; *CFH* complement factor H; *ELN* elastin; *HTRA1* HtrA serine peptidase 1; *IGFBP7* insulin-like growth factor binding protein 7; *LIPC* lipase-hepatic; *LOC389641* uncharacterized LOC389641; *LOXL1* lysyl oxidase-like 1; *PEDF* pigment epithelium derived factor; *POLR2B* polymerase (RNA) II (DNA directed) polypeptide B; *REST* RE1-silencing transcription factor; *SKIV2L* superkiller viralicidic activity 2-like; *TLR3* toll-like receptor 3; *TNFRSF10A* tumor necrosis factor receptor superfamily-member 10a

CI confidence interval; *nAMD* neovascular age-related macular degeneration; *OR* odds ratio; *PCV* polypoidal choroidal vasculopathy

the remaining 45 SNPs in CFH, VEGF, *C2*, *CFB*, *SKIV2L*, *CETP,* 8p21, 4q12, *ELN*, *LIPC*, *LPL*, *FGD6*, *ABCA1*, *ABCG1*, *PGF*, *TLR3*, *LOXL1,* and *PEDF.*

The similarity and difference of PCV and nAMD attracted the interest in investigating the genetic susceptibility between them. There was a large sample size study investigated the genetic variants of 34 AMD loci for PCV and nAMD in East Asians [75]. The results showed that PCV and tAMD were highly correlated (r_g = 0.69, $P = 4.68 \times 10^{-3}$) in genetic variants. Weaker association for PCV compared to nAMD was found at *ARMS2-HTRA1* and *KMT2E-SRPK2*. The different association

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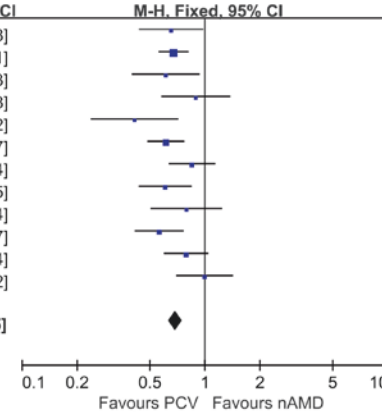
Study or Subgroup	PCV Events	PCV Total	nAMD Events	nAMD Total	Weight	Odds Ratio M-H, Fixed, 95% CI
Goto 2009 JPN	114	200	134	200	5.0%	0.65 [0.43, 0.98]
Hayashi 2010 JPN	558	1018	521	810	22.7%	0.67 [0.56, 0.81]
Lima 2010 Caucasian	35	110	319	736	4.9%	0.61 [0.40, 0.93]
Bessho 2011 JPN	144	238	86	136	3.7%	0.89 [0.58, 1.38]
Fuse 2011 JPN	56	120	68	100	3.4%	0.41 [0.24, 0.72]
Tanaka 2011 JPN	412	762	333	506	15.9%	0.61 [0.48, 0.77]
Yanagisawa 2011 JPN	235	396	229	362	8.4%	0.85 [0.63, 1.14]
Liang 2012 Chinese	199	324	226	312	7.7%	0.61 [0.43, 0.85]
Cheng 2013 Chinese	110	166	134	188	3.7%	0.79 [0.50, 1.24]
Zhang 2013 Chinese	307	500	232	314	9.5%	0.56 [0.41, 0.77]
Yoneyama 2014 b JPN	400	666	206	314	9.7%	0.79 [0.60, 1.04]
Woo 2015 Japanese	141	222	196	308	5.2%	0.99 [0.69, 1.42]
Total (95% CI)		**4722**		**4286**	**100.0%**	**0.69 [0.63, 0.75]**
Total events	2711		2684			

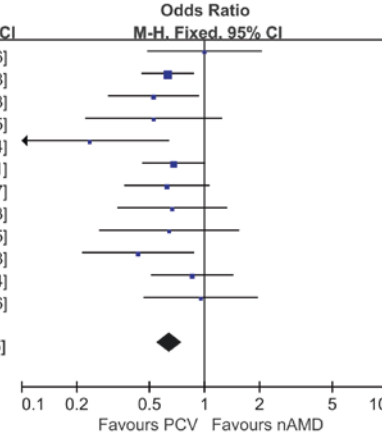
Heterogeneity: Chi² = 15.56, df = 11 (P = 0.16); I² = 29%
Test for overall effect: Z = 8.10 (P < 0.00001)



b

Study or Subgroup	PCV Events	PCV Total	nAMD Events	nAMD Total	Weight	Odds Ratio M-H, Fixed, 95% CI
Goto 2009 JPN	82	100	82	100	4.0%	1.00 [0.49, 2.06]
Hayashi 2010 JPN	387	509	338	405	24.4%	0.63 [0.45, 0.88]
Lima 2010 Caucasian	29	55	250	368	8.3%	0.53 [0.30, 0.93]
Bessho 2011 JPN	95	119	60	68	4.2%	0.53 [0.22, 1.25]
Fuse 2011 JPN	38	60	44	50	4.8%	0.24 [0.09, 0.64]
Tanaka 2011 JPN	287	381	207	253	16.6%	0.68 [0.46, 1.01]
Yanagisawa 2011 JPN	156	198	155	181	9.3%	0.62 [0.36, 1.07]
Liang 2012 Chinese	139	162	136	151	5.4%	0.67 [0.33, 1.33]
Cheng 2013 Chinese	70	83	84	94	3.3%	0.64 [0.27, 1.55]
Zhang 2013 Chinese	213	250	146	157	7.2%	0.43 [0.21, 0.88]
Yoneyama 2014 b JPN	275	333	133	157	8.5%	0.86 [0.51, 1.44]
Woo 2015 Japanese	96	111	134	154	4.1%	0.96 [0.47, 1.96]
Total (95% CI)		**2361**		**2138**	**100.0%**	**0.64 [0.54, 0.75]**
Total events	1867		1769			

Heterogeneity: Chi² = 9.61, df = 11 (P = 0.57); I² = 0%
Test for overall effect: Z = 5.35 (P < 0.00001)



c

Study or Subgroup	PCV Events	PCV Total	nAMD Events	nAMD Total	Weight	Odds Ratio M-H, Fixed, 95% CI
Goto 2009 JPN	32	100	52	100	5.8%	0.43 [0.24, 0.77]
Hayashi 2010 JPN	171	509	183	405	22.0%	0.61 [0.47, 0.80]
Lima 2010 Caucasian	6	55	69	368	2.6%	0.53 [0.22, 1.29]
Bessho 2011 JPN	49	119	26	68	3.2%	1.13 [0.61, 2.08]
Fuse 2011 JPN	18	60	24	50	3.0%	0.46 [0.21, 1.02]
Tanaka 2011 JPN	125	381	126	253	16.6%	0.49 [0.36, 0.68]
Yanagisawa 2011 JPN	79	198	74	181	7.6%	0.96 [0.64, 1.45]
Liang 2012 Chinese	60	162	90	151	9.5%	0.40 [0.25, 0.63]
Cheng 2013 Chinese	40	83	50	94	4.0%	0.82 [0.45, 1.48]
Zhang 2013 Chinese	94	250	86	157	10.7%	0.50 [0.33, 0.75]
Yoneyama 2014 b JPN	125	333	73	157	10.1%	0.69 [0.47, 1.02]
Woo 2015 Japanese	45	111	62	154	5.0%	1.01 [0.62, 1.66]
Total (95% CI)		**2361**		**2138**	**100.0%**	**0.62 [0.55, 0.71]**
Total events	844		915			

Heterogeneity: Chi² = 21.75, df = 11 (P = 0.03); I² = 49%
Test for overall effect: Z = 7.39 (P < 0.00001)

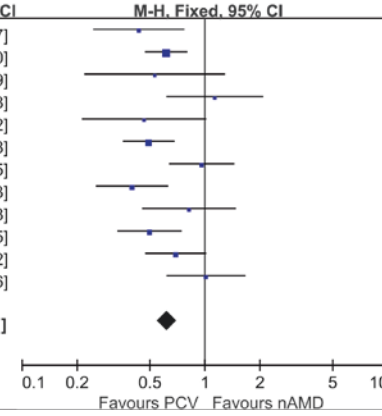


Fig. 8.2 Forest plots of meta-analysis comparing the SNP rs10490924 at *ARMS2* between PCV and nAMD. (**a**) allele frequencies; (**b**) dominant model; (**c**) recessive model

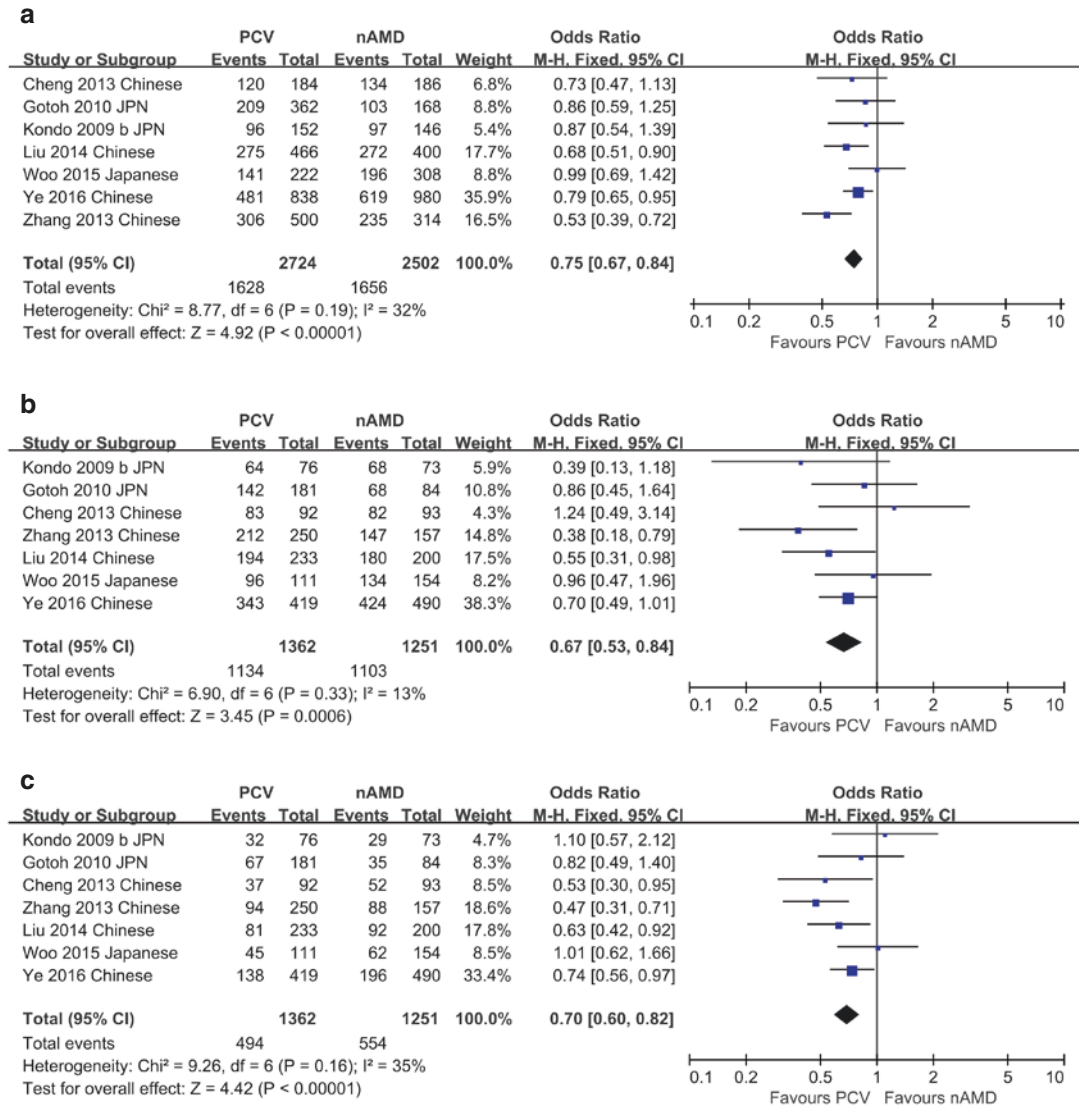


Fig. 8.3 Forest plots of meta-analysis comparing the SNP rs11200638 at *HTRA1* between PCV and nAMD. (**a**) allele frequencies; (**b**) dominant model; (**c**) recessive model

of *ARMS2-HTRA1* variants between PCV and nAMD was confirmed in this meta-analysis. But *KMT2E-SRPK2* was investigated in only one study and therefore no meta-analysis was performed. In 2016, an article using exome sequencing identified a rare variant, rs77466370, in *FGD6* was significantly associated with PCV (OR = 2.12) but not with CNV (OR = 1.13) [70]. Our meta-analysis confirmed that most genetic polymorphisms were distributed similarly between nAMD and PCV, But also some polymorphisms had a statistically significant difference between PCV and nAMD. These results suggest that PCV and nAMD have shared the majority of genetic background, while the differences of *ARMS2-HTRA1* locus and *FGD6* variants may be correlated with the differences in the pathologic and clinical manifestations of PCV and nAMD. The molecular mechanisms underlying their differences in pathogenesis remain to be further investigated.

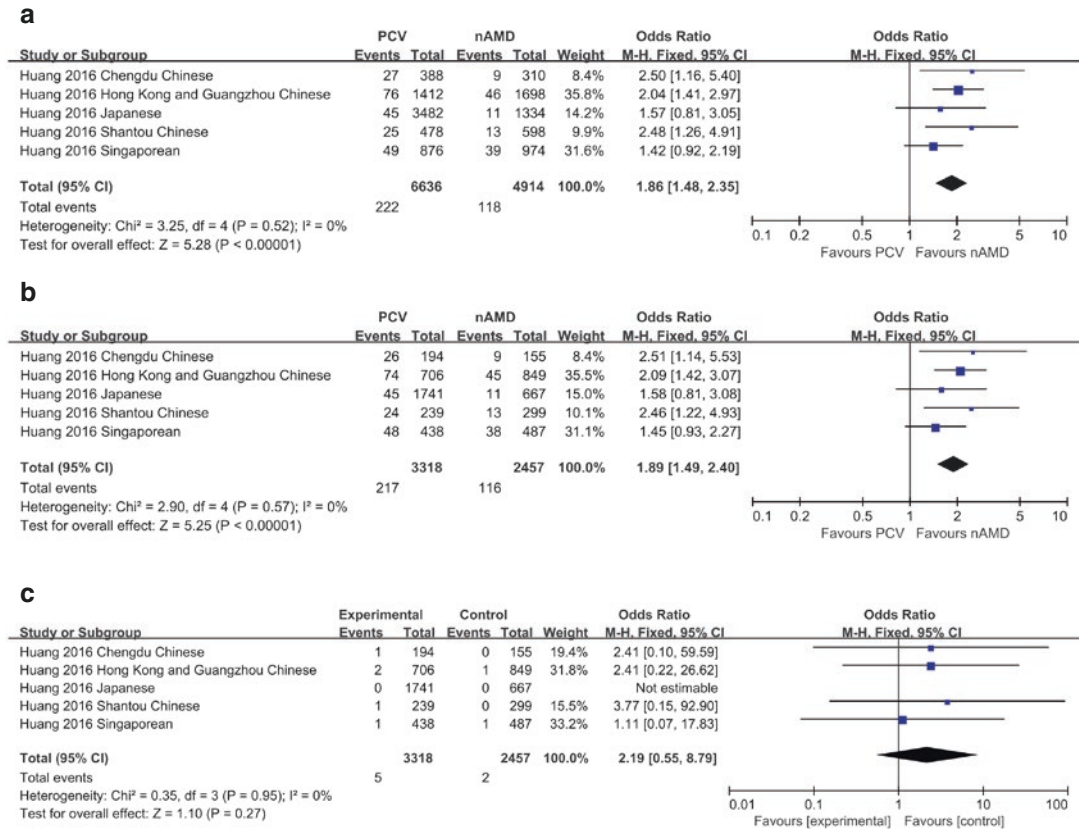


Fig. 8.4 Forest plots of meta-analysis comparing the SNP rs77466370 at *FGD6* between PCV and nAMD. (**a**) allele frequencies; (**b**) dominant model; (**c**) recessive model

ARMS2-HTRA1 locus located at chromosome 10q26. It was one of the most strong associated locus with AMD [79, 80]. There are many SNPs in this locus and they are in strong linkage disequilibrium. ARMS2 was expressed in the mitochondria of the outer segment of photoreceptors [81]. The function of *ARMS2* was suggested to be associated with loss of function of RPE [81]. HTRA1 can inhibit transforming growth factor-βin chronic inflammation [82]. In the *HTRA1* transgenic mice model, retinal pigment epithelium atrophy, photoreceptor degeneration, and grape-cluster structure in choroidal vasculature were reported, which is similar to the PCV phenotype [83]. However, our meta-analysis found that the effect size of *ARMS2-HTRA1* locus was weaker in PCV compared to nAMD. Further functional studies are needed to elucidate the role of *ARMS2-HTRA1* locus in PCV/nAMD.

FGD6 located at chromosome 12q22. FGD6 expresses in all human tissue but has a higher level of expression in retina and choroid, especially in retinal microvascular endothelial cells. Rs77466370, c.986A > G (p.Lys329Arg), is a rare variant with the minor allele frequency of 0.02–0.03 in normal subjects. FGD6-Arg329 has a different pattern of intracellular localization from FGD6-Lys329. In vitro, FGD6 could promote endothelial cells tube formation, furthermore, FGD6-Arg329 promoted more abnormal vessel development in the mouse retina than FGD6-Lys329 [70]. These functional studies support the role of FGD6 in the pathogenesis of PCV.

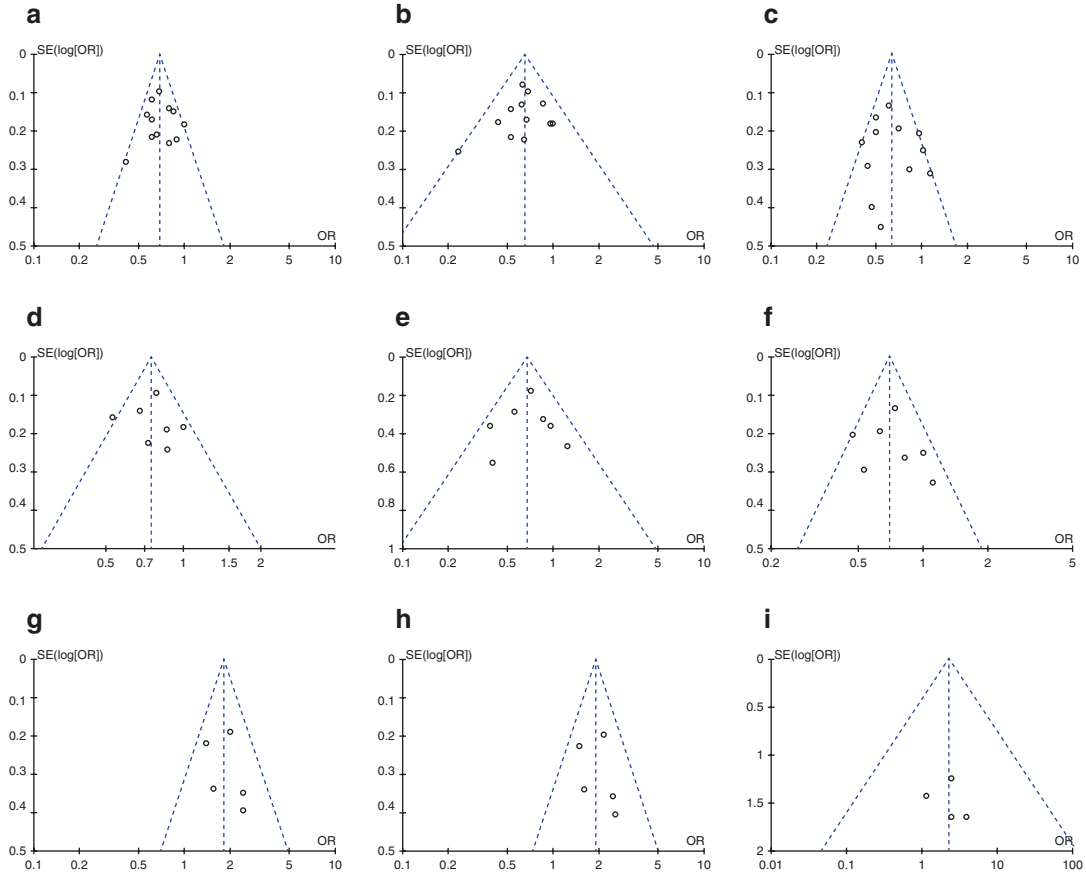


Fig. 8.5 Funnel plots of the meta-analysis comparing rs10490924 (**a**–**c**), rs11200638 (**d**–**f**) and rs77466370 (**g**–**i**) between PCV and nAMD. (**a**, **d**, **g**) Allele frequencies; (**b**, **e**, **h**) dominant model; C, F, I. recessive model

8.5 Summary

In summary, we pooled the results 57 SNPs in 20 genes that had been investigated in both PCV and nAMD in the same studies. Among them, 11 SNPs at the *ARMS2-HTRA1* locus and rs77466370 in *FGD6* showed significant differences between PCV and nAMD, but the other SNPs had similar distribution between PCV and nAMD. Our results suggest that PCV and nAMD have shared the majority of genetic components, but the variants distributed differently between these two conditions may explain the pathogenic and clinical differences of PCV and nAMD.

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9 Genetic Epidemiology of Quantitative Traits of Primary Open Angle Glaucoma

Sahil Thakur, Xiaoran Chai, and Ching-Yu Cheng

Abstract

Nowadays, GWAS meta-analysis is the preferred approach for gene discovery in genetic studies to evaluate disease pathogenesis. This chapter comprehensively reviews the genetic epidemiology of primary open angle glaucoma-related endophenotypes such as optic disc parameters, including vertical cup-to-disc ratio (VCDR), optic disc area (DA) and cup area (CA), or risk factors such as intraocular pressure (IOP) and central corneal thickness (CCT).

Keywords

GWAS · POAG · IOP · CCT · VCDR · RNFL Genes

S. Thakur · X. Chai
Singapore Eye Research Institute, Singapore National Eye Centre, Singapore, Singapore
e-mail: thakur.sahil@seri.com.sg; chai.xiaoran@seri.com.sg

C.-Y. Cheng (✉)
Singapore Eye Research Institute, Singapore National Eye Centre, Singapore, Singapore

Duke-NUS Medical School, Singapore, Singapore

Department of Ophthalmology, Yong Loo Lin School of Medicine, National University of Singapore and National University Health System, Singapore, Singapore
e-mail: chingyu.cheng@duke-nus.edu.sg

Primary open angle glaucoma (POAG) is the most common cause of irreversible blindness in the world [1]. The management of the disease requires lifelong follow-up due to the chronic nature of the disease. Glaucoma patients hence make up approximately one-fourth of the outpatient activity in eye hospitals across the world [2]. This number is likely to increase given the proportion of a rapidly ageing population and an increase in the number of patients with glaucoma [1, 3]. In spite of glaucoma being a significant public health problem, the exact pathogenesis of glaucoma is not fully understood. Among the risk factors for glaucoma, positive family history has been recognised as an important risk factor. Early studies have shown that first-degree relatives of glaucoma patients have an estimated ten times risk of glaucoma as compared to the general population [4].

In order to better understand the pathological basis of the complex diseases, it is imperative to begin by investigating whether susceptibility to the disease has a genetic basis and evaluating the magnitude and type of this susceptibility. Once a genetic component is established, the next step is to search for the genes that cause or contribute to the disease. The two main approaches for this evaluation are linkage and association. Traditional linkage analysis based studies have subsequently found out several glaucoma causing mutations in genes like myocilin (*MYOC*), optineurin (*OPTN*), glutathione

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S-transferase mu-1, WD repeat-containing protein 36 (*WDR36*), cytochrome P450 subfamily I polypeptide 1 (*CYP1B1*), neurotrophin 4 (*NTF4*), ankyrin repeat and SOCS box-containing protein 10 (*ASB10*) and TANK-binding kinase 1 (*TBK1*) [5–12]. This approach is however limited by its candidate gene approach based on prior knowledge and is unsuitable to evaluate pathophysiology of a complex polygenic disease like glaucoma.

Genes that cause diseases, however can have different variations, such as single nucleotide polymorphisms (SNPs) and copy number variations (CNVs). The pathogenic effect of the genetic variations can also vary from weakly to strongly pathogenic or even protective. Thus an alternative to conventional linkage analysis i.e. genome-wide association studies (GWAS), have been commonly used for glaucoma genetic studies. GWAS is used to compare the genetic profile of SNPs across the entire genome in affected cases and unaffected controls. This is to evaluate the association of a particular genomic region with a certain trait or disease. GWAS tests more than a million SNPs for association with a disease phenotype or other trait in order to adequately cover the genome. Subsequently, statistical tests are performed using a multiple testing correction threshold significance of 5E-8 (rather than $P < 0.05$) after adjusting for correlated and linked SNPs. Then a replication study is performed in an independent cohort to verify the findings and to reduce false-positive findings. Earlier smaller GWAS used to lack statistical power to identify weaker signals with small genetic effects. Currently, meta-analysis, with much bigger sample size, is the most commonly used method for new gene discovery. It is vital to however consider, that most of the associated SNPs that are identified in GWAS studies are common variants, have small effect size and many unaffected people carry these risk alleles. Thus the results from these studies need to be carefully evaluated and interpreted for optimal clinical application. Recently several GWAS studies focussed on the associations between SNPs and intermediate traits associated with glaucoma as these traits are less complex than interpreting the disease as a whole.

The complex pathogenesis of glaucoma can be thus be reduced by studying quantitative traits of glaucoma, such as optic disc parameters, including vertical cup-to-disc ratio (VCDR), optic disc area (DA) and cup area (CA), or risk factors such as intraocular pressure (IOP) and central corneal thickness (CCT). These traits are defined as endophenotypes as they are associated with glaucoma in the population, are heritable, can be found in an individual whether or not the disease is present, and cosegregate, to an extent, with the disease [13].

Heritability is used to quantify the genetic component of endophenotypes. It ranges from 0 (no genetic effect) to 1 (a phenotype that is completely determined by genes). The heritability of glaucoma endophenotypes is moderate to high: 0.72 for DA, between 0.48 and 0.66 for VCDR, 0.55 for IOP and 0.85 for CCT [14]. Thus the study of these endophenotypes has its advantages. These traits being quantitative continuous variables and are less likely to be misclassified, unlike the diagnosis of glaucoma which is a binary variable. They can be studied in patients without glaucoma and have a simpler genetic basis than the disease itself. This strategy also allows individuals to be ranked using genetic risk scores as opposed to ranking individuals as patients and controls. This is especially important in a chronic disease like glaucoma as any control may later become a case. Additionally commingling analysis demonstrated that the presence of a single major gene accounts for 18% of the variance of IOP in a population but could not give evidence of a single major genetic determinant for CCT [15, 16]. This highlights the importance of finding these genes that contribute to the expression of the phenotype like IOP.

Nowadays, GWAS meta-analysis is the preferred approach for novel gene discovery in genetic studies. Additionally, sophisticated statistical approaches offer ways to interpret large data sets with more chances of uncovering vari-

ants with small effect sizes. In this chapter, we highlight the genes associated with glaucoma endophenotypes and how this information has improved our understanding of the disease. We additionally review the epidemiology of these traits to highlight the global variation in the expression of these genes. Finally, we explore the potential therapeutic applications and future trends in genomic research.

9.1 Intraocular Pressure

IOP is the most important risk factor in the development of glaucoma especially POAG. Additionally being the only modifiable risk factor for POAG; reduction of IOP has been shown to retard the progression of the disease in glaucoma cases with and without elevated IOPs. Population-based studies have shown a 16% increased risk of glaucoma for every mm Hg increase in IOP [17]. Thus as a reliably measurable endophenotype, IOP is an ideal target for GWAS studies. Initial GWASs identified many genetic loci associated with IOP, including *GAS7, TMCO1, GLCCI1-ICA1, ADAMTS18-NUDT7, FOXP1, FAM125B* and *ARHGEF12* [18–22]. Meta-analysis of large populations has identified additional genomic regions, such as *CAV1-CAV2, ABO, MYOC, LMX1B, LMO7, NR1H3, FNDC3B, RAPSN, PKHD1, ADAMTS8, HIVEP3, ANTXR1, AFAP1, ARID5B, FOXO1* and *INCA1* as additional IOP related loci [23–26].

With the availability of larger data sets in 2018, three large studies have come out with even more loci that are associated with IOP. In a study on over 100,000 patients of the UK Biobank, Gao et al. identified 671 genotypic variants associated with IOP, additionally, 103 of these are novel loci. The novel IOP genes are *LMX1B, NR1H3, MADD* and *SEPT9*. This study has been able to account for 40.4% of IOP heritability from all genotyped variants while the GWAS significant variants explained 7.2%. This study highlights the polygenic and pleiotropic nature of the IOP loci [27]. Khawaja et al., evaluated a combined cohort of 139,555 participants from UK Biobank, EPIC Norfolk and 14 other studies from the International Glaucoma Genetics Consortium (IGGC), reporting a total of 112 loci with 68 novel loci. These variants collectively explain 17% of IOP variance in the EPIC Norfolk cohort and 9% in the UK Biobank. These loci discovered by this study suggest a strong role for angiopoietin receptor tyrosine kinase signalling, lipid metabolism, mitochondrial function and developmental processes underlying risk for elevated IOP. In order to evaluate the relationship between these IOP and POAG, they additionally tested for association of the discovered loci in a clinically diagnosed cohort of 3853 POAG cases/ 33,480 controls and self-reported (1500 cases/331,078 controls). In total, 14 SNPs were significantly associated and showed a correlation between the effect sizes for IOP and POAG [28]. Likewise, using data from UK Biobank and IGGC, MacGregor et al. reported 101 SNPs for IOP, and 85 of these were novel. They utilised these data to derive an allele score based on the IOP loci and loci influencing optic nerve head morphology. They then evaluated this score in another Australian cohort of 1734 people with advanced glaucoma and 2938 controls. Individuals in the top 5%, 10% and 20% of the allele scores were at significantly higher risk of POAG relative to the bottom 5%, 10% and 20%, respectively (OR = 7.8, 5.6 and 4.2, respectively) [29]. This highlights how IOP endophenotype associated genomic data can be utilised in developing a genetic test for glaucoma that can help in early diagnosis.

Additionally, discovery of new genes is adding insight into molecular pathway-based pathogenesis of POAG, like genes associated with extracellular matrix metabolism (ECM), transforming growth factor-β (TGF-β) signalling, tumour necrosis factor α (TNF-α) signalling, vascular tone maintenance, rho kinase-associated pathway and the regulation eye development pathway. Table 9.1 summarises the GWAS studies that have helped in identifying loci that are associated with IOP.

Table 9.1 Summary of GWAS studies for intraocular pressure

Population	No. of subjects		New Loci	References	Year
	Discovery	Replication			
Netherland/UK, Australia, Canada, Blue Mountain	11,972	7482	*GAS7, TMCO1*	Van Koolwijk et al. [18]	2012
European	6236		*TMCO1*	Ozel et al. [19]	2013
Australia/UK	2175	4866	7p21 near *GLCCI1, ICA1*	Blue Mountain Eye Study [20]	2013
UK/European	2774	22,789	*FAM125B (MVB12B)*	Nag et al. [21]	2014
European, Asian	27,558, 7738	4284 cases, 95,560 controls	*CAV1/CAV2,* chromosome 11cluster (*RAPSN, PTPRJ*), *FDNC3B, ABCA1, ABO*	Hysi et al. [23]	2014
European	8105	7471	*ARHGEF12*	Springelkamp et al. [22]	2015
European, Latino, Asian, and African/ European, Asian	69,756	37,930	40 novel loci, with 14 showing replication in the validation set. New loci like *HIVEP3, AFAP1, ARID5B, COL6A1, FOXO1, GLIS3, EFEMP1, CAV2, INCA, ANTXR1* and *LPP*	Choquet et al. [26]	2017
European, Asian	37,930		*ADAMTS8*	Springelkamp et al. [25]	2017
Australian, European	3071 cases, 6750 controls	3853 cases, 33,480 controls	*MYOF/CYP26A1, LINC02052/ CRYGS, LMX1B* and *LMO7*	Gharahkhani et al. [24]	2018
European	133,492	11,018 cases and 126,069 controls	Evaluated 85 novel loci, confirmed 53 in the replication set	MacGregor et al. [29]	2018
European	115,486	Springelkamp et al. summary statistics	*LMX1B,NR1H3, MADD and SEPT9* with 103 novel loci discovery	Gao et al. [27]	2018
European	103, 382	36,173	68 novel loci, Total IOP loci 112, includes *LR1G1, DGKG, ANKH, EXOC2, PKHD1, PDE7B, CTNBP2, FXO32, LMX1B, ME3, ETS1, VPS13C, CDH11* and *FANCA*	Khawaja et al. [30]	2018

9.2 Central Corneal Thickness

CCT is a highly heritable ocular quantitative trait with up to 95% of its phenotypic variance due to genetics [31]. The evaluation of glaucoma-related traits with GWAS has led to the discovery of several genes associated with this endophenotype in the past few years. GWAS, conducted in European, Asian and Latino populations, have identified SNPs in or nearby *AKAP13, COL5A1, COL8A2, FAM53B, FOXO1, IBTK, LRRK1, RXRA-COL5A1* and *ZNF469* to be associated with CCT [32–36]. A meta-analysis consisting of European and Asian individuals subsequently identified 16 additional loci associated with CCT, including *LPAR1* and *ARID5B* [37]. Additionally, *WNT7B* has also been found as a locus for CCT in Latinos and South Indians [38, 39]. Iglesias et al. conducted a large scale cross-ancestry GWAS of over 25,000 individuals of European and Asian descent. They reported additional novel loci for CCT from the cohort near *LTBP1, STAG1, ARL4C, NDUFAF6, ADAMTS8, DCN* and *POLR2A*. What is interesting to note is however that several SNPs that they reported are lying in close proximity to genes linked with Mendelian disorders like Fuchs endothelial dystrophy (*COL8A2, AGBL1*), Loeys-Dietz syndrome (*TGFB2,*

SMAD3), Ehler-Danlos Syndrome (*ADAMTS2, COL5A1*), cornea plana (*DCN-KERA*), Marfan syndrome (*FBN1*) and Brittle cornea syndrome (*ZNF469*) [40]. These loci still however can only explain around 8% of CCT heritability [37, 40]. Nevertheless with increasing evidence from GWAS studies, the role of CCT as an endophenotype in glaucoma is becoming clearer. The role of biological pathways like the collagen and ECM (*ADAMTS2, ADAMTS8, COL5A1, ZNF469, COL8A2, COL6A2, COL12A1, FBN1, LOXL2, LUM/DCN/KERA, THSB2*), TGF-β signalling (*FBN1, FNDC3B, TGFB2, LTBP1*), binding processes (*ARVCF, STAG*), coagulation and fibrinolysis systems (*HABP2*), endocytic machinery (*STON2*), skeletal morphogenesis (*RUNX2*), embryonic development and cell growth (*FGF1*) and mitochondrial processes (*NDUFAF6*) in the disease process has also been explored [33, 37, 40]. Table 9.2 summarises the GWAS studies that have helped in identifying loci that are associated with CCT.

9.3 Optic Disc Parameters

Glaucoma-related optic disc parameters include, but are not limited to vertical cup disc ratio (CDR), optic disc area (DA), cup area (CA) and rim area (RA). Since all these parameters measure the optic disc, they have been found to be inter-correlated. The CA has a much higher coefficient of variation than the RA and appears to drive the majority of variation in CDR. The CDR is also highly correlated with the CA (correlation coefficient = 0.89) and is the parameter most relevant clinically. The DA conversely is the sum of the CA and RA, both the disc rim and disc cup correlations are also high (correlation coefficient > 0.6) [41]. Thus we look at these traits together to understand the association of these endophenotypes with glaucoma. Many loci for quantitative optic nerve parameters have been identified. Several are associated with more than one optic nerve parameter: *CDC7/*

Table 9.2 Summary of GWAS studies for central corneal thickness

Population	No of subjects		New Loci	Reference	Year
	Discovery	Replication/ association			
European	1445	824	*COL5A1, AKAP13* and *AVGR8*	Vitart et al. [32]	2010
Australian, European	5058		*FOX01, ZNF469*	Lu et al. [33]	2010
Asian	5080	7349	*COL8A2, RXRA-COL5A1*	Vithana et al. [34]	2011
Asian	7711		*IBTK, CHSY1,* intergenic regions of 7q11.2 and 9p23	Cornes et al. [35]	2012
European	3931	1418	Confirmed *RXRA-COL5A1* and *ZNF469*	Hoehn et al. [36]	2012
Asian, European	>20,000		16 novel loci, including *COL4A3, FNDC3B, TBL1XR1, NR3C2, VKORC1L1, LPAR1, ARID5B, ARHGAP20, GLT8D2, SMAD3* for both cohorts	Lu et al. [37]	2013
Latino	3584	931	*WNT7B* and confirmed *NR3C2, IBTK, LPAR1, RXRA-COL5A1, COL5A1, FOXO1, ARHGAP20, LRRK1, ZNF469*	Gao et al. [38]	2016
South Indian	195		*WNT7B, DSC2, MIR622, MTHFD1L*	Fan et al. [39]	2018
European, Asian	17,803, 8107	5008 cases/ 35,472 controls for POAG	*LTBP1, STAG1, ARL4C, NDUFAF6, ADAMTS8, DCN* and *POLR2A* but could not find correlation between POAG and CCT	Iglesias et al. [40]	2018

TGFβR3 and *CARD10* are associated with DA and CDR, *CDKN2B/CDKN2B-AS1, CHEK2, HSF2, COL8A1, SSSCA1, SIX1/SIX6, BMP2* and *RERE* are associated with CA and CDR; and *ATOH7, SALL1* and TMTC2 are associated with DA, CA and CDR [42–44]. Gharahkhani et al. recently showed that an integrative approach using meta-analysis of GWAS summary statistics from POAG and its correlated traits (VCDR, CA, DA and IOP) is capable of identifying new risk loci by increasing the study statistical power [24]. Multiple GWASs have previously identified loci associated with more than one optic disc parameter and also POAG. However, the limitation of currently available GWAS data is that most of the data are from the European populations. Replication of loci like *CDC7- TGFβR3, ATOH7, COL8A1, CDKN2B/CDKN2BAS, BMP2* and *CHEK2* in Asian and Latino population has subsequently shown that different ethnicities share common intermediates in glaucoma etiopathogenesis [25, 44–46] It however appears that several loci have not yet been replicated due to inter-ethnic variations or sample size limitations. With the availability of larger datasets and newer integrative approaches to GWAS data interpretation, more loci are likely to be identified in the coming years. Using GWAS from optic disc parameters, pathways like cell cycle arrest (*CDKN1A, CDKN2B/CDKN2A* and *CDC7*), retinal ganglion cell genesis (*ATOH7*) and eye development (*SIX6*) have been postulated to play a part in glaucoma development [24, 43, 44, 46]. Building on this knowledge researchers are developing multi-locus genetic risk scores based on top SNPs in these associated genes which can be ultimately adopted as a genetic test for glaucoma in the coming years [30, 43]. Table 9.3 summarises the GWAS studies that have helped in identifying loci that are associated with optic disc parameters.

9.4 Retinal Nerve Fibre Layer Thickness

As glaucoma is a neurodegeneration disease, retinal nerve fibre layer (RNFL) thinning is one of the most important changes related to either disease onset or progression. The retinal nerve fibre layer can also be easily measured by optical coherence tomography (OCT). The heritability for RNFL thickness is 0.48, which was estimated from 2620 people with mean age of 48 living in a small town in The Netherlands [47]. Given this heritability estimate, genetic factors appear to play an important role in RNFL thickness as well as glaucoma development. However, studies have uncovered fewer glaucoma risk genes related to RNFL thinning as compared to other glaucoma endophenotypes such as IOP, CDR and CCT (Table 9.4).

The first two loci (*DCLK3* and *SIX1*) showing suggestive association with RNFL thickness were identified in 2011 in the Dutch population [48]. The most popular RNFL thickness associated gene is *SIX6* that has been reported in several independent GWAS and experimental studies. The first insight of the *SIX6* missense variant (rs33912345) was given in 2014 [49]. The authors showed that the RNFL thickness of patients carrying homozygous *SIX6* risk allele (C allele of rs33912345) was significantly lower than patients carrying homozygous *SIX6* reference allele (A allele of rs33912345). They also provided a successful validation of this result in the zebra fish model. In the same year, another independent GWAS study conducted in the Singapore Chinese Eye Study discovered the same *SIX6* risk allele for RNFL thinning [50]. In this study, the author did not only evaluated the significance level of association, but also provided effect estimation of the risk allele in *SIX6* using additive linear model adjusting for age, gender, population stratification and axial length. The estimated effect size was about—1.4 μm in

Table 9.3 Summary of GWAS studies for optic disc parameters

Population	Subjects		Optic disc trait	New Loci	References	Year
	Discovery	Replication/ association				
Australian, European	1368, 848		DA, CA	*ATOH7, RFTN1*	Macgregor et al. [41]	2010
European	7360	4455	DA	*CDC7-TGFβR3, ATOH7, SALL1*	Ramdas et al. [42]	2010
			VCDR	*CDKN2B, SIX1, ATOH7, SCYL1, CHEK2, DCLK1, BCAS3, RERE, ARID3A*		
Asian, European	4445	9326	DA	*CARD10,* confirmed *CDC7-TGFβR3, ATOH7* in Asians	Khor et al. [46]	2011
European, Asian	21,094, 6784		VCDR	*COL8A1, DUSP1, EXOC2, PLCE1, ADAMTS8, RPAP3, SALL1, BMP2, HSF2, CARD10*	Springelkamp et al. [43]	2014
European/ Asian	17,248, 6841		DA	*CDC42BPA, F5, DIRC3, RARB, ABI3BP, DCAF4L2, ELP4, TMTC2, NR2F2, HORMAD2*	Springelkamp et al. [44]	2015
			CA	*DHRS3, TRIB2, EFEMP1, FLNB, FAM101, DDHD1, ASB7, KPNB1, BCAS3,TRIOBP*		
Latino	3586	941	VCDR	Confirmed *CDC7-TGFβR3, ATOH7, COL8A1, CDKN2B/ CDKN2BAS, BMP2, CHEK2* in Latinos	Nannini et al. [45]	2017
European/ Asian	23,899, 8373	6429 cases, 414,404 controls	VCDR	*RPE65, F5, PDZD2, RREB1, DGKB, VCAN, ENO4, PSCA,RBM23*	Springelkamp et al. [25]	2017
			CA	*CDC42BPA,CRISPLD1, FAM169B*		
			DA	*UGT8, CTNNA3, PRDM16, GADD45A, VGLL4, ASB7*		
			POAG	*CDKN1A*		
European/ Asian	3071 cases, 6750 controls	3853 cases, 33,480 controls	POAG/IOP and optic disc parameters	*MYOF, CYP26A1, LINC02052/CRYGS, LMX1B, LMO7*	Gharahkhani et al. [24]	2018

Table 9.4 Summary of genetic studies on retinal nerve fibre layer thickness

Population	Subjects		Loci	References	Year
	Discovery	Replication/ association			
Dutch population	1488		*DCLK3, SIX1*	Axenovich et al. [48]	2011
NEIGHBOUR/ GLAUGEN	262 cases/256 controls		*SIX6*	Ulmer Carnes et al. [49]	2014
Singapore Chinese	1243		*SIX6*	Cheng et al. [50]	2014
European	231		*SIX1-SIX6*	Kuo et al. [51]	2015
Japanese	756 cases/ 3094 controls		*CDKN2B*	Yoshikawa et al. [52]	2017
Japanese	2306		*SIX1-SIX6*	Yoshikawa et al. [55]	2017
Japanese	565 cases/1104 controls	607 cases/ 455 controls	*SIX6*	Shiga et al. [53]	2017

both patients with or without glaucoma. Another SNP (rs10483727) located in-between *SIX1* and *SIX6* was also identified to be significantly associated with RNFL in a European population with a smaller effect size of −0.16 μm [51]. This SNP is indeed in high linkage disequilibrium ($r^2 = 1$) with previously identified *SIX6* SNP (rs33912345). Another Japanese study performed a targeted association analysis to investigate the relationship between 26 tagging SNPs in *SIX1-SIX6* locus and 32 distinct regional circumpapillary retinal nerve fibre layer thicknesses (cpRNFLTs) sectors. As a result, only one significant association was detected between rs33912345 and cpRNFLT in the inferior region at 292.5°–303.8°. In addition, the authors also examined the relationship between cpRNFLT and rs10483727 which was not included in tagging SNPs and further confirmed that this well-known RNFL-associated SNP is actually associated with inferior region cpRNFLT at 281.3°–303.8°. In another study to evaluate the association of already reported four glaucoma-susceptible genes to cpRNFLTs and corresponding visual field defects, the *CDKN2B (AS1)* gene was associated with RNFL in the temporal region at 330°–360° and 0°–30°. These region-specific signals corresponded to visual field defects of the paracentral/lower hemifield ($P < 0.05$) indicating that functional loss in glaucoma corresponds to the presence of genetic risk variants [52]. Another study on genetic factors related to RNFL conducted in the Japanese population estimated a much bigger effect (−2.16 μm) of *SIX6* risk allele (rs33912345) on RNFL thickness [53]. Using *SIX6* risk allele to model the glaucoma condition, researchers were also able to discover *SIX6* risk allele induced gene expression changes, which may responsible for glaucoma development or progression [54]. With the availability of larger and well-characterised datasets like the UK Biobank, it is expected that more genetic associations for RNFL thickness will be uncovered in the next few years.

9.5 Integrating Endophenotypes with Glaucoma

Till date more than 35 validated POAG loci have been identified from GWAS studies. Table 9.5 summarises these loci. Optic disc parameters associated loci (*CDC7/TGFβR3, CDKN2B/ CDKN2B-AS1* and *SIX1/SIX6*), IOP associated loci (*CAV1/CAV2, ABCA1, ARHGEF12, ANKH, LMO7, DGKG, EXOC2, PDE7B* and *GAS7*), CCT associated loci (*FNDC3B*) and RNFL-associated loci (*SIX1/SIX6 and CDNK2B*) are also important POAG associated loci (Fig. 9.1).

This overlap and review of pathological processes and intermediate association of endophenotype genes indicate that many of these loci are regulators in pathways implicated in glaucoma pathogenesis [68]. The integration of GWAS data from endophenotypes has helped highlight several pathways that can potentially be targeted as treatment avenues for glaucoma. The poten-

Table 9.5 Published genome-wide association studies of primary open angle glaucoma

Population	New loci	References	Year
Japanese	*ZP4, PLXDC2, TMTC2*	Nakano et al. [56]	2009
Japanese	*SRBD1, ELOVL5*	Meguro et al. [57]	2010
Iceland	*CAV1/CAV2*	Thorliefsson et al. [58]	2010
Australian	*CDKN2BAS, TMCO1*	Burdon et al. [59]	2011
US European	*SIX1/SIX6, 8q22*	Wiggs et al. [60]	2012
Japanese	*CDKN2BAS,SIX1/SIX6*	Osman et al. [61]	2012
Australian	*ABCA1, AFAP1, GMDS*	Gharahkhani et al. [62]	2014
Asian	*ABCA1, PMM2*	Chen et al. [63]	2014
Multiethnic	*TGFβR3, FNDC3B*	Li et al. [64]	2015
European	*ARHGEF12*	Springelkamp et al. [22]	2015
US European	*TXNRD2, ATXN2, FOXC1, GAS7*	Cooke Bailey et al. [65]	2016
Australian-European	*MYOF/CYP26A1, LINC02052/ CRYGS, LMX1B, LMO7*	Gharahkhani et al. [24]	2018
Asian	*LHPP, HMGA2, MEIS2, MAP3K1, LOXL1*	Shiga et al. [66]	2018
Multiethnic	*FMNL2, PDE7B, TMTC2, IKZF2, CADM2, DGKG, ANKH, EXOC2*	Choquet et al. [67]	2018

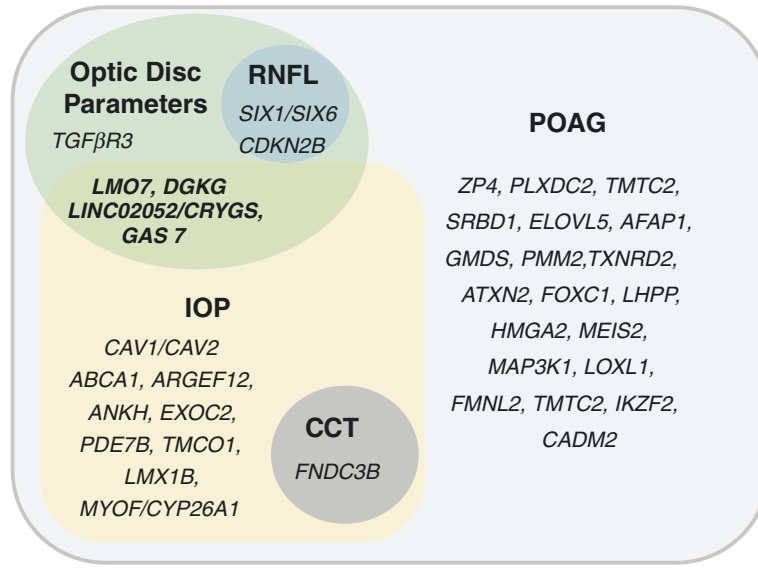

Fig. 9.1 Relationship between primary open angle glaucoma and glaucoma-related endophenotype genes identified via GWAS

tial use of short hairpin *CAV1* and *CAV2* silencing and control lentiviruses to modulate outflow capacity of trabecular meshwork has already been demonstrated [69]. GWAS data from patients with congenital glaucoma shows the additive role of partial gene mutations. The evidence comes from angiopoietin-*TEK* signalling pathway-based studies that show anterior chamber development is dependent on *TEK* gene dosage. In childhood glaucoma, *TEK* mutations have autosomal dominant transmission pattern with a variable expression that can range from normal to disease variants [70]. There are other glaucoma associated genes like *FOXC1, MYOC, SIX6* and *ANGPT1* which also contribute to the early onset of glaucoma [71]. Studies to evaluate the association between Mendelian linkage studies identified glaucoma genes and rarer variants identified with GWAS are generating new opportunities to investigate the cellular and molecular mechanisms

behind the disease. The biggest challenge ahead is however to perform functional validation and detailed analysis of the in vivo function of these GWAS identified genes in the eye. This requires a lot of experimental data based on existing animal models and the development of several new gene knockout models. Only after carefully combining human genetic and metabolomics data with animal and computational models, we can combine several lines of evidence that can establish candidate genes for glaucoma therapeutic purposes.

In conclusion, these are exciting times for researchers involved in GWAS analysis of glaucoma and glaucoma-related traits. With the availability of larger data sets, advanced bioinformatics tools and innovative approaches to integrate already available data into concurrent research, future genetic studies appear to hold the key to unlock the complex mechanisms that underline glaucoma pathogenesis. Insight from these studies has potential role in glaucoma management by generating therapeutic strategies that can complement the reduction of IOP, and prevent blindness from this irreversible disease.

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Association Studies on Retina Diseases in Chinese Population

10

Li Gan, Bo Gong, and Zhenglin Yang

Abstract

Age-related macular degeneration (AMD) and diabetic retinopathy (DR), the leading causes of visual impairment in different ethnic groups, are known to be multifactorial and complex diseases with a strong genetic predisposition. In recent years, the advent of genome-wide association studies (GWAS) and whole-exome sequencing (WES) has greatly improved the screening and identification of genetic variants in these complex retinal diseases. In this chapter, we overviewed and summarized recent major advances in the association studies on AMD and DR in the Chinese population. Based on these studies, we found that the genetic variants in the *HTRA1, CFH, SKIV2L, CETP* genes were strongly associated with AMD. These genes including *HTRA1, CFH, CETP, ARMS2, C3, FGD6, ABCG1,* and *ANGPT2*, had significant associations with polypoidal choroidal vasculopathy (PCV), one type of AMD. There were also significant associations of several genes with DR in the Chinese population. These results further confirmed that genetic factors play a critical role in the development of these retina diseases.

Keywords

Association study · Retina diseases · Age-related macular degeneration (AMD) Chinese population · Diabetic retinopathy (DR)

10.1 Association Studies of Age-Related Macular Degeneration

Age-related macular degeneration (AMD), a progressive chronic disease of the central retina, is the most common cause of irreversible vision loss and blindness worldwide [1, 2]. As life expectancy increases, the rising prevalence of AMD has brought a socio-economic burden to individuals and the whole society. Currently, it has become a public health concern in China [3, 4]. Advanced AMD is broadly categorized as geographic atrophy (dry) and neovascular age-related macular degeneration (wet), which poses a risk to severe visual impairment in older adults. Polypoidal choroidal vasculopathy (PCV), a

L. Gan · B. Gong · Z. Yang (✉)
The Key Laboratory for Human Disease Gene Study of Sichuan Province and Department of Laboratory Medicine, Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, Sichuan, China

Institute of Chengdu Biology, Sichuan Translational Medicine Hospital, Chinese Academy of Sciences, Chengdu, Sichuan, China
e-mail: zhenglin.yang@hsc.utah.edu

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common subtype of neovascular AMD, is characterized by polypoidal lesions in the choroidal vasculature [5, 6]. Multiple environmental factors and genetic predisposition play a critical role in AMD development. Cigarette smoking and older age are the major risk factors for AMD pathogenesis [7–10]. In addition, obesity and cardiovascular diseases are also associated with an increased risk of AMD [11–13]. However, the etiology of AMD remains poorly understood. In recent years, genome-wide association studies (GWAS) and whole-exome sequencing (WES) have been widely used to analyze the contribution of genetic variations to complex diseases such as AMD and PCV [14] (Table 10.1).

In 2006, Dewan et al. and Yang et al. reported that the same SNP rs11200638 in the promoter region of *HTRA1* was strongly associated with wet AMD in a Chinese cohort and a Caucasian cohort, respectively. *HTRA1*, a major genetic risk factor for wet AMD, played a key role in AMD susceptibility [2, 15]. An association analysis was followed by Chen et al. and found that three SNPs of the *CFH* gene, rs1329428, rs800292, and rs3753394, carried an increased risk for exudative AMD in the Chinese population [16]. In 2010, four SNPs in *CFH*, including rs3753394, rs800292, rs106170, and rs1329428, were reported to be significantly associated with wet AMD among Han Chinese in mainland China [17]. In 2013, another study in a Chinese cohort of 165 AMD patients and 216 controls validated and found that two complement pathway genes, *CFH* (rs800292 and rs1410996) and *C2/CFB* (rs9332739 and rs4151667), contributed to AMD [18]. Since 2013, several replication studies were conducted to investigate the association of rs429608 in *SKIV2L* with AMD in China. Liu et al., Lu et al. and Ye et al. found a strong association between *SKIV2L* rs429608 and AMD [4, 19, 20]. In 2015, a genome-wide association study (GWAS) was conducted across multiple sites in East Asia, including 6345 exudative AMD cases and 15,980 controls [21]. In this study, *CETP* rs2303790, an East Asian-specific mutation, was reported to be strongly associated with increased risk of AMD (OR = 1.70, $P = 5.60 \times 10^{-22}$). *C6orf233*, *SLC44A4,* and *FGD6* were identified as new AMD loci in East Asians, two of which (*SLC44A4* and *FGD6*) were coding, non-synonymous variants. The results of the combined meta-analysis showed that *C6orf233* rs2295334, *SLC44A4* rs12661281, and *FGD6* rs10507047 all reached the threshold of genome-wide significant ($P < 5 \times 10^{-8}$), providing new evidence for the genetic mechanisms of AMD. Later, Huang et al. performed a whole-exome sequencing study on 3988 neovascular AMD cases and 8495 controls, and identified a missense mutation, rs7739323, which is located in the *UBE3D* gene. *UBE3D* rs7739323 was significantly associated with AMD risk in East Asian populations (OR = 0.74, $P = 1.46 \times 10^{-9}$), indicating an underlying role in nAMD pathogenesis [22]. In 2016, SNPs rs2268615 and rs2268614 in *PGF* were reported to be significantly correlated with nAMD in the Chinese population [23]. Recently, Huang et al. replicated the genetic association between coding and *UTR* variants and wet AMD. SNP rs189132250 in *BBX* and rs144351944 in *FILIP1L* were found to be associated with wet AMD in a Chinese cohort [24].

Several association studies of polypoidal choroidal vasculopathy (PCV), one type of AMD, were also conducted in China. In 2008, Lee et al. performed an association analysis in a Chinese cohort and found evidence of the association between *CFH* variants (rs3753394 and rs800292) and *HTRA1* variants (rs11200638) and PCV [25]. Later, 11 SNPs of *CFH* were reported to be associated with an increased risk of PCV, suggesting that the complement pathway played an important role in the pathogenesis of PCV [26]. In 2011, Zhang et al. found that rs10757278 on 9p21 was significantly associated with PCV in a Chinese Han population [27]. Furthermore, there was reported a significant association of PCV with *ARMS2* rs10490924 in a Chinese Han cohort [28]. In 2013, SNP rs5882 in the cholesteryl ester transfer protein (*CETP*) gene was strongly related to PCV ($P = 2.73 \times 10^{-4}$) [29]. In 2014, Liu et al. and Meng et al. confirmed a significant association between *CETP* rs3764261 and PCV in a Chinese study ($P = 4.04 \times 10^{-4}$ and 0.0247, respectively) [30, 31]. All results indicated that high-density lipoprotein (HDL) metabolic path-

Table 10.1 The contribution of genetic variations to retinal diseases such as AMD, PCV, and DR were analyzed by Genome-wide association study (GWAS) and exome sequencing in Chinese population

Authors	Years	Sample size		Disease	Ethnicity	Method	SNP	Chr	Related gene	OR	*P* value
		Case	Control								
Cheng et al.	2015	6345	15,980	AMD	East Asian	GWAS	rs2303790	16	*CETP*	1.70	5.60×10^{-22}
							rs2295334	6	*C6orf223*	0.78	6.19×10^{-18}
							rs12661281	6	*SLC44A4*	1.27	1.08×10^{-11}
							rs10507047	12	*FGD6*	0.87	2.85×10^{-8}
Huang et al.	2015	3988	8495	AMD	East Asian	WES	rs7739323	6	*UBE3D*	0.74 (0.63–0.88)	1.46×10^{-9}
Huang et al.	2016	194	1253	PCV	Han Chinese	WES	rs77466370	12	*FGD6*	3.46	6.11×10^{-8}
							rs10490924	10	*ARMS2/HTRA1*	1.95	1.17×10^{-9}
							rs3753396	1	*CFH*	1.82	9.31×10^{-8}
							rs1065489	1	*CFH*	1.81	1.28×10^{-7}
Sheu et al.	2013	570	437	DR(T2D)	Chinese	GWAS	rs9565164	13	*TBC1D4-COMMD6-UCHL3*	1.70	1.3×10^{-7}
							rs1399634	2	*LRP2-BBS5*	1.50	2.0×10^{-6}
							rs2380261	2	*ARL4C-SH3BP4*	1.50	2.1×10^{-6}
Huang et al.	2011	174	675	DR(T2D)	Chinese	GWAS	rs2811893	1	*MYSM1*	1.50 (1.03–2.20)	3.09×10^{-7}
							rs12092121	1	*MYSM1*	1.50 (1.03–2.20)	3.09×10^{-7}
							rs13163610	5	*Unknown*	3.59 (1.36–9.47)	3.22×10^{-15}
							rs17376456	5	*Unknown*	3.63 (1.38–9.58)	2.99×10^{-15}
							rs1571942	10	*PLXDC2*	1.67 (1.06–2.65)	3.47×10^{-7}
							rs12219125	10	*Unknown*	1.62 (1.02–2.58)	9.29×10^{-9}
							rs4838605	10	*ARHGAP22*	1.58	1.87×10^{-9}
							rs11101355	10	*ARHGAP22*	1.65	8.92×10^{-7}
							rs11101357	10	*ARHGAP22*	1.65	8.92×10^{-7}
							rs2038823	13	*HS6ST3*	2.33 (1.13–4.77)	4.68×10^{-11}

AMD age-related macular degeneration; *PCV* polypoidal choroidal vasculopathy; *DR* diabetic retinopathy; *T2D* type 2 diabetes mellitus; *GWAS* genome-wide association study; *WES* whole-exome sequencing; *Chr* chromosome; *OR* odds ratio

way may be involved in PCV pathogenesis. Additionally, *C3* rs17030 was found to be robustly associated with PCV (OR = 2.94, $P = 0.008$) [32]. In 2016, a whole-exome sequencing study was performed in a Han Chinese cohort of 194 PCV cases and 1253 control individuals [5]. In this study, three SNPs reached the significance threshold of $P < 4.24 \times 10^{-7}$, including one SNP in the *ARMS2*/*HTRA1* locus (rs10490924), and two SNPs in the *CFH* gene (rs3753396 and rs1065489), all of which were robustly associated with PCV and CNV. Moreover, a missense variant in the *FGD6* gene, rs77466370, was identified as significantly associated with PCV (OR = 3.46, $P = 6.11 \times 10^{-8}$) but not with AMD (OR = 1.38, $P = 0.37$). Recently, Ma et al. reported that two new susceptibility genes, *ABCG1* (rs225396) and *ANGPT2* (rs4455855 and rs13269021), were associated with PCV [33, 34].

10.2 Association Studies of Diabetic Retinopathy

Diabetic retinopathy (DR), a common and specific microvascular complication of diabetes mellitus, is the leading cause of vision loss in working-age population around the world [35, 36]. The etiology and mechanism of DR are considered to be complex and multifactorial. Furthermore, duration of diabetes, systolic blood pressure (SBP), urinary albumin, and glycemic control have consistently been identified as major risk factors for the progression of diabetic retinopathy [37–39]. Recently, the Lifeline Express Diabetic Retinopathy Screening Program, carrying out a multi-hospital-based cross-sectional study across mainland China (both southern and northern), determined the prevalence and risk factors associated with diabetic retinopathy in the Chinese population. In these investigations, the age-gender-standardized prevalence of DR was 27.9% (95% CI, 27.2% to 28.6%), similar to that in other population-based studies from Western countries [40]. Both genetic and epigenetic factors play important roles in diabetic retinopathy development [41, 42].

Diabetic retinopathy is a multifactorial disease. Genome-wide association studies (GWAS) have been widely employed in the field of diabetic retinopathy genetics [43]. A total of two GWAS have been conducted on diabetic retinopathy in the Chinese population (Table 10.1). The first study was performed to identify the susceptibility genes that increase the risk of DR in a Chinese cohort of 174 DR cases and 575 controls [44]. In this study, significant associations with DR were identified in five novel loci: *MYSM1*, *PLXDC2*, *ARHGAP22*, *HS6ST3*, and an unknown gene on chromosome 5q. The SNPs rs2811893 and rs12092121, located in the *MYSM1* gene on chromosome 1, were associated with a 1.50-fold increase in DR risk ($P = 3.09 \times 10^{-7}$). *PLXDC2* (rs1571942) and *ARHGAP22* (rs4838605, rs11101355, and rs11101357) were found to be correlated with associated with DR, which were involved in endothelial cell angiogenesis and increased capillary permeability. The SNP rs2038823, an intronic region of the *HS6ST3* on 13q, was related to DR (OR = 2.33, $P = 4.68 \times 10^{-11}$). And rs12219125 on chromosome 10p was also associated with DR (OR = 1.62, $P = 9.29 \times 10^{-9}$). Another genome-wide association study identified three novel loci: *TBC1D4-COMMD6-UCHL3* (rs9565164, $P = 1.3 \times 10^{-7}$), *LRP2-BBS5* (rs1399634, $P = 2.0 \times 10^{-6}$), and *ARL4C-SH3BP4* (rs2380261, $P = 2.1 \times 10^{-6}$) in the Chinese discovery cohort of 1007 individuals. These genetic regions were involved in insulin regulation, inflammation, lipid signaling and apoptosis pathways, which were possibly associated with DR. [45] In addition, Hu et al. verified a significant association of SNP rs39059 in *CPVL*/*CHN2* with diabetic retinopathy in Chinese type 2 diabetic patients [46]. In 2015, the association of *KCNJ11* rs5219 with diabetic retinopathy was replicated in the Chinese Han population with T2DM [47]. Another Chinese study involving 618 cases and 400 controls confirmed a significant association between *CRP* rs2808629 and DR, with an OR of 1.296($P = 0.006$) [48]. Later, a replication analysis revealed that rs17684886 in *ZNRF1* and rs599019 near *COLEC12* were associated with diabetic retinopathy and that rs6427247 near *SCYL1BP1* and rs899036 near *API5* were associated with the risk of severe diabetic retinopathy in

the Chinese population [49]. In 2016, Cheung et al. performed the cross-sectional case-control study to validate the associations of the GWAS identified DR-associated SNPs with severe DR in Chinese patients with T2DM. SNP rs2115386, an intronic SNP in the *INSR*, was strongly associated with severe DR and supported the role of insulin resistance in the pathogenesis of DR [50]. Recently, Jin and colleagues found evidence of the association between SNP rs955333 on 6q25.2 and diabetic retinopathy in the Chinese population [51].

Compliance with Ethical Requirements Li Gan, Bo Gong, and Zhenglin Yang 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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11 Congenital Stationary Night Blindness (CSNB): An Inherited Retinal Disorder Where Clear Correlations Can Be Made

Christina Zeitz, Juliette Varin, and Isabelle Audo

Abstract

Congenital stationary night blindness (CSNB) refers to a group of clinically and genetically heterogeneous retinal disorders. Few of those are associated with fundus abnormalities while the majority show largely normal fundi. Clear genotype-phenotype correlations can be performed for patients with the Riggs-form of CSNB, fundus albipunctatus, Oguchi disease, and the Schubert-Bornschein-form of CSNB. In total 15 different genes were associated with those showing more than 500 different mutations in more than 400 cases. While mutations in genes important for the rod phototransduction lead to the Riggs-form of CSNB, fundus albipunctatus, Oguchi disease, mutations in genes important for the downstream signaling from the photoreceptors to the adjacent bipolar cells lead to the Schubert-Bornschein-form of CSNB. In this book chapter, phenotypic characteristics of the different forms of CSNB are summarized for an accurate diagnosis. Clear genotype-phenotype correlations mentioned herein should lead to an improvement of genetic testing.

Keywords

CSNB · Full field electroretinogram (ffERG) Fundus · Riggs-type ERG · Oguchi disease Fundus albipunctatus · Schubert-Bornschein-type ERG · Incomplete CSNB · Complete CSNB · Major gene defects · Genotype-phenotype correlations · Protein localization correlates with the phenotype · In vitro and in vivo models

11.1 Introduction

Congenital stationary night blindness (CSNB) is a clinically and genetically heterogeneous inherited retinal disorder. This book chapter aims to summarize the main and common features of the disease. As the name implicates the disease is

C. Zeitz (✉) · J. Varin
Department of Genetics, Sorbonne Université, INSERM, CNRS, Institut de la Vision, Paris, France
e-mail: christina.zeitz@inserm.fr, juliette.varin@inserm.fr

I. Audo
Department of Genetics, Sorbonne Université, INSERM, CNRS, Institut de la Vision, Paris, France

CHNO des Quinze-Vingts, INSERM-DGOS CIC 1423, Paris, France

Institute of Ophthalmology, University College of London, London, UK
e-mail: Isabelle.Audo@inserm.fr

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present from birth. However, other clinical symptoms are not always reflected by the name: night blindness may not be the chief symptom and is a very subjective sign in the well-lighted environment of big cities. Similarly, not all cases show a stationary disease; progression can be also noted. In many cases diurnal vision is also affected: reduced visual acuity, light sensitivity, high myopia, nystagmus, and strabismus may be also diagnosed. However, using fundus examination and electroretinography, patients can be precisely clinically diagnosed, classified which will direct the genetic strategy. Patients, for whom a genetic analysis does not identify a known gene defect, may harbor mutations in non-coding regions of known genes underlying the same phenotype or in a novel gene. For the latter ones, the respective protein localization can be as well correlated to the phenotype.

11.2 Epidemiology

To our knowledge, the frequency of CSNB in the general population has not been documented. This might be due to undiagnosed cases. Indeed, specific clinical examinations are necessary to correctly diagnose CSNB. In 2015 we summarized genetic data of 300 index patients with CSNB, previously published by us and others [1]. Taking into account our newly collected cases since then, we see that these numbers are continuously growing. To date (February 2019), in total, more than 500 different mutations have been published. Similarly, since 2015 in more than 180 novel index cases with CSNB from our worldwide collaboration, the genetic cause was resolved. In respect to the collection of our large European cohort with inherited retinal disorders, including ~5000 index cases, 2% of those present CSNB.

11.3 Clinical Features

To correctly diagnose CSNB, fundus examination and full-field electroretinogram (ffERG) incorporating the International Society for Clinical Electrophysiology of Vision (ISCEV) standards are essential [2]. Furthermore, documentation of the mode of inheritance is important for the proper classification of CSNB. Table 11.1 summarizes the main clinical features of the different forms of CSNB.

11.3.1 Riggs-Type of Congenital Stationary Night Blindness: A Form of Night Blindness with Largely Normal Fundus

The Riggs-type of CSNB [4] represents a rod-photoreceptor dysfunction. The ffERG shows severely reduced scotopic responses. At low light intensities (dark adaptation (DA) 0.01) the b-wave is severely reduced or absent. At a bright flash in addition to the b-wave reduction also the a-wave is reduced (DA 10.0). This reflects primary rod-dysfunction. Photopic ERGs (LA 3.0 and LA 3.0 30 Hz) are normally consistent with normal cone function. This form of CSNB has been reported as autosomal dominant and autosomal recessive modes of inheritance with specific mutations in genes coding for proteins of the rod phototransduction cascade. The phenotype is relatively mild including night blindness no nystagmus, and normal photopic visual acuity with only a few cases showing myopia [1, 5, 6]. This relatively mild phenotype may be the reason why to date only few cases with this Riggs-type of CSNB were described. Historically, this form of CSNB was detected in the Nougaret family, coming from Southern France, [7–11], and in another family reported by Rambusch [12, 13]. In both,

Table 11.1 Summary of general clinical characteristics and mode of inheritance of most CSNB (modified from [3])

	Riggs-CSNB	Funds albipunctatus	Oguchi	icCSNB	cCSNB	*GNB3*-CSNB
Mode of inheritance	Autosomal dominant autosomal recessive	Autosomal recessive	Autosomal recessive	x-chromosomal autosomal recessive	x-chromosomal autosomal recessive	Autosomal recessive
Fundus abnormalities	No	Variable, dots focal lesions from the retinal pigment epithelium/Bruch's membrane complex to the inner limiting membrane with an additional decrease in outer nuclear layer thickness	Mizuo-Nakamura	Myopia possible	Myopia	No
Night blindness	Yes	Yes	Yes	Possible	Yes	Possible
Photophobia	No	No	No	Often	No	Possible
High myopia	No	No	No	Possible	Often	No
Hyperopia	No	No	No	Often	No	No
Nystagmus	No	No	No	Often	Often	Possible
Strabismus	No	No	No	Often	Often	No
DA 0,01 ERG	⇓	⇓	⇓	↓	⇓	↓
DA 3,0 ERG	a-wave ⇓ b-wave ⇓	a-wave ⇓ b-wave ⇓	a-wave ⇓ b-wave ⇓	a-wave normal b-wave ⇓	a-wave normal b-wave ⇓	a-wave normal b-wave ↓
LA 3,0 ERG	Relative normal	Mildly abnormal	Relative normal	a-wave normal but broader b-wave ⇓	a-wave normal but broader b-wave with a sharply rising peak	a-wave normal but can be broader b-wave can be ↓
LA 3,0 30 Hz ERG	Relative normal	Mildly abnormal	Relative normal	⇓	Normal amplitude, may have a flattened trough, may show mild implicit time shifts	↓ not as icCSNB
Recovery of ERG after long dark adaption	No	Yes	Yes	No	No	

⇓ strongly reduced or absent, ↓reduced

the phenotype was transmitted as an autosomal dominant trait. A few cases with autosomal recessive Riggs-type CSNB have been reported. However, in the latter cases especially the photopic responses are less consistent with the classic Riggs-form of CSNB [14–16].

11.3.2 Fundus Albipunctatus: A Form of Night Blindness with Fundus Abnormalities

Fundus albipunctatus (FA) is characterized indirectly by rod-photoreceptor dysfunction. Albeit that the respective gene defect underlying this disease is expressed in the retinal pigment epithelium, the mutant form leads to the dysfunction of the recycling of rhodopsin, specifically expressed in rod-photoreceptors. Therefore patients are effectively "bleached" most of the time. Thus the diagnosis cannot be made purely by ISCEV standard ERGs as the recovery following extended DA needs to be confirmed [1]. At low light intensities (DA 0.01) the b-wave is severely reduced or absent. At a bright flash in addition to the b-wave reduction also the a-wave is reduced (DA 10.0), which reflects primary rod-dysfunction. Similar scotopic ERGs are found in patients with the Riggs-form of CSNB. However, in most patients, unlike Riggs-type CSNB, prolonged dark adaptation typically results in significant or complete recovery of rod-mediated ERG amplitudes although there is phenotypic variability [17]. Photopic ERGs are mildly abnormal in about half of the cases and often show flicker ERG delay [1]. In addition patients with FA are characterized by night blindness but visual acuity, color vision, and visual fields are usually normal. Strikingly, patients with FA have specific fundus abnormalities. They often show small white dots in the posterior pole and mid-periphery with sparing of the macular region. Fundus appearance may change with time from flecks in childhood to fine dots with age that may fade or increase over the years [1, 18–20]. Albeit FA does not present a progressive rod-cone dystrophy showing optic nerve pallor, nor retinal blood vessel attenuation, nor pigmentary bone spicule migration in the periphery, phenotypic variability leading to more progressive phenotypes have been described [17, 21]. The disease is inherited in an autosomal recessive fashion. Albeit only one gene defect is associated with this disease, founder mutations in the same gene are responsible that this form is a relatively frequent cause of CSNB [1].

11.3.3 Oguchi Disease: A Form of Night Blindness with Fundus Abnormalities

Oguchi disease (OD) is also characterized by rod-photoreceptor dysfunction. Similar scotopic ERGs are found in patients with the Riggs-form of CSNB. At low light intensities (DA 0.01) the b-wave is severely reduced or absent. Also here, in response to a bright flash in addition to the b-wave reduction also the a-wave is reduced (DA 10.0), which reflects primary rod-dysfunction. After prolonged dark adaptation, rod sensitivity recovers, and the ERG response to a single-flash results in nearly normal a- and b-waves [22]. However, unlike FA, the ERG response to a subsequent single bright flash is markedly attenuated and similar to that recorded after short dark adaptation (20 min). The abnormal desensitization of the rod system to a repeated bright flash is caused by continued activation of the phototransduction cascade by rhodopsin molecules. This continues until all the chromophore is recycled, requiring a further extended period of DA [1, 23]. Photopic recordings are usually normal [24]. Patients affected with OD are congenitally night blind, but have normal visual acuity, color vision, and visual fields [1]. Similarly as in patients with FA, patients with OD show specific fundus abnormalities, known as the Mizuko-Nakamura phenomenon: the fundus has a golden-yellow discoloration that disappears after prolonged dark adaptation [25, 26]. Although Oguchi disease is considered to be a stationary and rela-

tively mild disease, some cases show more severe phenotypes and disease progression [27–32]. Historically OD was first described by a Japanese soldier complaining of night blindness. The disease is inherited in an autosomal recessive mode of inheritance, with only a few cases described.

11.3.4 Schubert-Bornschein-Type of Congenital Stationary Night Blindness a Form of "Night Blindness" with Largely Normal Fundus

The Schubert-Bornschein-type of CSNB represents a signaling defect from photoreceptors to bipolar cells. Similarly, as the Riggs-type of CSNB, the ffERG show severely reduced scotopic responses. At low light intensities (dark adaptation (DA) 0.01) the b-wave is reduced or absent. However, unlike in the Riggs-type of CSNB, in the Schubert-Bornschein-type of CSNB, after stimulation with a bright flash, only the b-wave is reduced while the a-wave is normal (DA 10.0), resulting in an electronegative waveform [33]. The Schubert-Bornschein-type of CSNB is the most common form of CSNB with largely normal fundi. It can be further subdivided into an incomplete (ic) and complete (c) form of CSNB. This classification is based on ffERG characteristics [34, 35] but is also in correlation with the localization of the proteins implicated in CSNB [1].

11.3.5 Incomplete Congenital Stationary Night Blindness

The incomplete form of CSNB (icCSNB) is characterized by both ON- and OFF-bipolar cell dysfunction. The ffERG shows reduced but present scotopic responses to a dim flash. Therefore this form was called incomplete CSNB [1]. At low light intensities (DA 0.01) the b-wave is reduced but present. At a bright flash, only the b-wave is reduced, while the a-wave is normal (DA 10.0), confirming normal rod phototransduction. This results in the previously mentioned electronegative ERG waveform [34]. The photopic responses are severely affected: the LA 3.0 30 Hz ERG is severely reduced and delayed with most having a distinct bifid peak. The single-flash cone ERG (LA 3.0) is also markedly subnormal with a profoundly reduced b/a ratio such that the a- and b-wave are usually of similar size [1]. Long-duration stimulation shows abnormalities in both ON- and OFF-responses [36]. Incomplete CSNB gets sometimes misdiagnosed with cone dystrophy due to profound photopic alteration, but the macula is usually normal unlike in cone dystrophies [1, 37]. However, in some cases, disease progression and more severe phenotypes were noted [38–42]. The incomplete form is a common form of CSNB and has been mainly reported in X-linked and in a few autosomal recessive cases with mutations in genes coding for proteins present at the synapse of photoreceptors. The phenotype of icCSNB is more heterogeneous than the one observed of cCSNB (please see below) with patients present with little or no night vision disturbances [35, 43–45]. However, photophobia is more common in icCSNB [44]. In addition, icCSNB patients may have myopia, hyperopia, nystagmus, strabismus, reduced visual acuity, and color vision defects [44].

11.3.6 Complete Congenital Stationary Night Blindness

The complete form of CSNB (cCSNB) is characterized by selective ON-bipolar cell dysfunction. The ffERG show severely reduced or absent scotopic responses to a dim flash. Therefore this form was called complete CSNB [1]. At low light intensities (dark adaptation (DA) 0.01) the b-wave is absent. At a bright flash only the b-wave is reduced, while the a-wave is normal (DA 10.0), confirming normal rod phototransduction. This results in the previously mentioned electronegative ERG waveform [34]. The photopic responses are less altered in cCSNB com-

pared to icCSNB: the LA 3.0 30 Hz ERG is often of normal amplitude but it has a pathognomonic, although it may have a flattened trough and may show mild implicit time shifts. The single-flash cone ERG (LA 3.0) has a normal a-wave amplitude but with a broadened through; the waveform has a sharply arising b-wave with no oscillatory potentials and a mildly reduced b/a ratio [34, 46]. Long-duration stimulation shows selective abnormalities in the ON-responses [36]. Similarly, as the incomplete form of CSNB, the complete form of CSNB is also a common form of CSNB with reported X-linked and autosomal recessive reported cases with mutations in genes coding for proteins mainly present at the dendritic tips of ON-bipolar cells. Patients with cCSNB are indeed congenitally night blind, have decreased visual acuity, and often show myopia, nystagmus and strabismus [1, 44]. Disease progression has not been noted.

11.3.7 GNB3-CSNB

Recently a novel gene defect underlying CSNB was identified [47, 48]. The phenotype cannot be classified in one of the subforms mentioned above [3]. Only a few cases have been described so far and the phenotypes seem to be variable even in those. At low light intensities (DA 0.01) the b-wave is reduced. At a bright flash, only the b-wave is reduced, while the a-wave is normal (DA 10.0), confirming normal rod phototransduction. The photopic responses are very variable: the LA 3.0 30 Hz ERG can be reduced and delayed. In the single-flash cone ERG (LA 3.0) the a-wave is normal but can be delayed and the b-wave is reduced and delayed. Long-duration stimulation shows abnormalities of the ON- but not the OFF-responses. Patients with mutations in *GNB3* may be night blind, showing myopia and nystagmus. But these ocular features were not observed in all patients. More patients with the same gene defect to be identified in the future may help to better classify this novel form of CSNB.

11.4 Molecular Biology

Table 11.2 summarizes the major gene defects underlying CSNB.

11.4.1 Gene Defects Implicated in Congenital Stationary Night Blindness

Inherited retinal disorders are clinically and genetically very heterogeneous. While often it is difficult to deliver clear genotype-phenotype correlations, for CSNB it is possible. Indeed, mutations in genes important for the rod phototransduction cascade can lead to isolated rod-photoreceptor dysfunction as found in the Riggs-form of CSNB, in FA and OD (Fig. 11.1). In contrast, mutations in genes important for the signaling from photoreceptors to bipolar cells or in genes important for the uptake of this signal lead to incomplete and complete CSNB, respectively. In vitro and in vivo models are in most cases helpful models to dissect retinal signaling and the pathogenic mechanisms implicated in CSNB [1]. Table 11.2 summarizes the different gene defects underlying CSNB, their chromosomal localization, the mode of inheritance, and the link to OMIM. Figure 11.1 shows the retinal localization of the molecules implicated in CSNB in a schematic drawing.

11.4.2 Gene Defects Underlying the Riggs-Type of Congenital Stationary Night Blindness, Fundus Albipunctatus, and Oguchi Disease

Specific mutations in genes coding for proteins important for the rod phototransduction cascade, including *RHO* coding for rhodopsin, *GNTA1*, coding for the α-subunit of transducin, *PDE6B*, coding for the β-subunit of the phosphodiesterase and *SLC24A1*, coding for the solute carrier family 24 members 1 have been identified in autosomal

Table 11.2 Gene defects of CSNB

Disease	OMIM	Mode of inheritance	Gene defect	OMIM	Localization
Riggs-CSNB CSNB1D # 613830 Autosmal recessive SLC24A1 #603617 15 q22.31	CSNBAD1 #610445	Autosomal dominant	*RHO*	#180380	3q22.1
	CSNBAD3 #610444	Autosomal dominant	*GNAT1*	#139330	3p21.31
	CSNB1G #616389	Autosomal recessive	*GNAT1*	#139330	3p21.31
	CSNBAD2 #163500	Autosomal dominant	*PDE6B*	#180072	4p16.3
Fundus albipunctatus	Fundus albipunctatus #136880	Autosomal recessive	*RDH5*	#601617	12q13.2
Oguchi	Oguchi disease 1 #258100	Autosomal recessive	*SAG*	#181031	2q37.1
	Oguchi disease 2 # 613411	Autosomal recessive	*GRK1*	#180381	13q34
Schubert-Bornschein icCSNB	CSNB2A # 300071	X-linked	*CACNA1F*	#300110	Xp11.23
Schubert-Bornschein icCSNB	CRSD[a] # 610427	Autosomal recessive	*CABP4*	#608965	11q13.2
Schubert-Bornschein icCSNB[b]	Retinal cone dystrophy 4 #610478	Autosomal recessive	*CACNA2D4*	#608171	12p13.33
Schubert-Bornschein cCSNB	CSNB1A #310500	X-linked	*NYX*	#300278	Xp11.4
Schubert-Bornschein cCSNB	CSNB1B #257270	Autosomal recessive	*GRM6*	#604096	5q35.3
Schubert-Bornschein cCSNB	CSNB1C #613216	Autosomal recessive	*TRPM1*	#603576	15q13.3
Schubert-Bornschein cCSNB	CSNB1E #614565	Autosomal recessive	*GPR179*	#614515	17q12
Schubert-Bornschein cCSNB	CSNB1F #615058	Autosomal recessive	*LRIT3*	#615004	4q25
GNB3-CSNB	CSNB1H #617024	Autosomal recessive	*GNB3*	#139130	12p13.31

[a]CRSD = congenital non progressive cone rod synaptic disorder
[b]Patient with this gene defect were previously diagnosed with icCSNB

dominant and autosomal recessive patients with the Riggs-type of CSNB [1]. The Nougaret family from the South of France had the p.Gly38Asp mutation in *GNAT1* [11]. In the meanwhile, two other *GNAT1* missense mutations were found in two autosomal dominant families [49, 50] and a homozygous *GNAT1* missense mutation in one autosomal recessive family [16], while the Rambusch family had the p.His258Asn mutation in *PDE6B* [51]. To date, only a second autosomal dominant family with a mutation in *PDE6B* was found [52]. Similarly, only a few autosomal dominant families revealed mutations in *RHO* [53–57] and a few autosomal recessive families revealed mutations in *SLC24A1* [14, 15]. The exact pathogenic mechanism of these mutations in genes coding for proteins of the phototransduction cascade, remains to be elucidated. Among others, constitutive activation would indeed explain the desensitization and reduced photo-response leading to night blindness [1].

Specific mutations in genes coding for proteins important for the rod phototransduction cascade, including *RDH5*, coding for the retinol dehydrogenase, *SAG* coding for arrestin and *GRK1* coding for the rhodopsin kinase have been identified

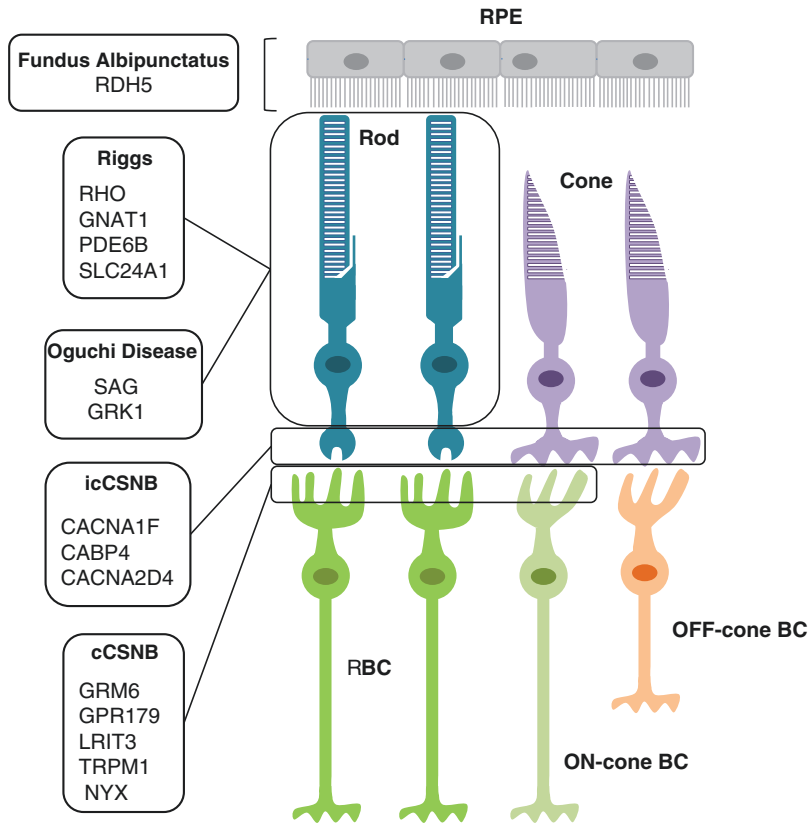

Fig. 11.1 Cellular role of proteins implicated in CSNB. Fundus Albipunctatus is due to mutations in *RDH5* and the respective protein is localized in the retinal pigmented epithelium (RPE, in gray). Mutations in genes coding for proteins localized in rod-photoreceptors (in blue), such as RHO, GNAT1, PDE6B, SLC24A1, SAG, and GRK1 can either cause the Riggs-type of CSNB or Oguchi disease. The icCSNB phenotype is attributable to defects in genes coding for proteins localized at the synapse of both rod- and cone photoreceptors (CACNA1F, CABP4, CACNA2D4) while cCNSB is due to mutations in *GRM6, GPR179, LRIT3, TRPM1, NYX* coding for proteins involved in the ON-BC processing (RBC, strong green and ON-cone BC, light green) while OFF-cone bipolar cells (OFF-cone BC, orange) do not present these proteins

in patients with autosomal recessive FA (*RDH5*) and OD (*SAG* and *GRK1*) showing some similarities with patients with the Riggs-form of CSNB but having additional fundus abnormalities. As mentioned before specific phenotypes can be recovered after extended DA. This correlates with the function of the affected proteins. Indeed, RDH5 is responsible for converting 11-*cis*-retinol into 11-*cis*-retinal in the retinal pigment epithelium (RPE), and is thus involved in the recycling of rhodopsin. Thus rhodopsin regeneration is delayed, FA patients are effectively "bleached" but after long DA rhodopsin levels can be normalized and thus the ERG [1]. OD patients have mutations in *SAG* and *GRK1*, both genes encoding proteins involved in the deactivation process of the phototransduction cascade [58, 59]. The phenotype represents basically no shut-off of the phototransduction cascade. After extended DA the ERG and fundus phenotype can be restored.

Gene defects underlying the Schubert-Bornschein-type of congenital stationary night blindness a form of "night blindness" with largely normal fundus.

11.4.3 Gene Defects Underlying Incomplete Congenital Stationary Night Blindness

Mutations in *CACNA1F*, coding for the α1-subunit (Cav1.4) of an L-type voltage-dependent calcium channel, *CABP4* coding for the calcium-binding protein 4, and *CACNA2D4* coding for the calcium channel, voltage-dependent, α-2/δ subunit 4 lead to icCSNB or related cone rod dystrophies with some overlapping phenotypes [1, 60–63]. The mutation spectrum comprises missense and splice site mutations, large and small deletions, and duplications. More recently we showed that intronic and synonymous variants in *CACNA1F* can also lead to a splice defect causing icCSNB [64]. The respective proteins are important downstream of the phototransduction cascade, by transmitting signals from the photoreceptors to the adjacent bipolar cells. Indeed, they localize at the photoreceptors and more specifically in a horseshoe-shaped manner in rod and cone photoreceptor synapse active zone within the outer plexiform layer (OPL) [1, 65–67]. Together these molecules are important for the correct functioning of the calcium channel. During darkness calcium ions are taken up by this channel, leading to glutamate release at the synaptic cleft [1]. Together, Cav1.4, CABP4, and CACNA2D4 form the pore are important to correctly targeting the channel to the synaptic membrane, to modulate calcium currents, and to bind calcium ions [1, 68–73]. Mutations in *CACNA1F*, *CABP4,* and *CACNA2D4* can be associated with loss or gain of function with insufficiently expressed genes resulting in an altered or non-functional calcium channel activity disturbing the regulation of the glutamate at the synaptic cleft. Different pathogenic mechanisms have been associated with the different mutations in these genes, which may explain the phenotypic variability. Both rod and cones make synaptic contacts with bipolar cells. There are two types of bipolar cells: ON- and OFF-bipolar cells expressing different glutamate receptors and responding differently to light. ON-bipolar cells express the metabotropic glutamate receptor 6 (GRM6/mGluR6) [74–76] and depolarize in response to light [77–79], while OFF-bipolar cells express ionotropic glutamate receptors and hyperpolarize at light offset [80–82]. ON-bipolar cells make synaptic contacts with both rod and cone photoreceptors, while OFF-bipolar cells only contact with cone photoreceptors [83]. Since molecules implicated in icCSNB localize in synaptic terminals of both, rod and cones, as a consequence ON- and OFF-responses in those patients are altered as shown in the ERG by long-duration stimulation.

11.4.4 Gene Defects Underlying Complete Congenital Stationary Night Blindness

Mutations in *GRM6*, coding for metabotropic glutamate receptor 6, *GPR179*, coding for the G-protein coupled receptor 179, *LRIT3* coding for the leucine-rich repeat, Ig-like and transmembrane domains 3 protein, *NYX*, coding for nyctalopin and *TRPM1*, coding for the transient receptor potential cation channel subfamily M member 1 lead to cCSNB [84–91]. The mutation spectrum comprises missense and splice site mutations, large and small deletions, and duplications [1]. The respective proteins play their role in ON-bipolar cells by receiving the signals transmitted from the synaptic cleft. Indeed, they localize at the dendritic tips of ON-bipolar cells within the outer plexiform layer (OPL) and are important for the depolarization of ON-bipolar cells at light stimulation, leading to glutamate decrease and TRPM1 channel opening at the end of this cascade [77, 79, 86, 92–97]. Mutations in these molecules lead to the absence of the b-wave and of ON-responses as shown in the ERG by long-duration stimulation.

11.4.5 *GNB3*-Gene Defect

As mentioned above, the *GNB3* gene defect cannot be strictly classified in the different subforms of CSNB. Thus we did not include the protein localization of GNB3 in Fig. 11.1. GNB3 coding for the β-subunit of the G-protein heterotrimer (Gαβγ) is known to be expressed in cones and ON-bipolar cells and modulates ON-bipolar cell signaling and cone transducin function in mice [98]. Due to its expression in cones as well in ON-bipolar cells the dual phenotype associated with *GNB3* mutations maybe explained [47].

11.4.6 Laboratory

Genetic testing of CSNB patients is important for genetic counseling of patients and their families to distinguish from progressive retinal dystrophies with similar phenotypic features [1]. For example, night blindness is one of the first presenting signs of progressive rod-cone dystrophy also called retinitis pigmentosa. At a young age, patients may initially show normal or near-normal fundus appearance. Therefore in addition to accurate phenotyping, molecular confirmation of CSNB helps to correctly diagnose and counsel patients. CSNB patient with largely normal fundus, a Riggs-ERG, and autosomal dominant or autosomal recessive inheritance should be screened for mutations in *RHO* = *GNAT1* > *PDE6B* and *SLC24A1* > *GNAT1* respectively. For patients with an autosomal recessive mode of inheritance and FA, *RDH5* should be targeted, while patients with autosomal recessive CSNB and a phenotype suggestive of OD should be screened for mutations in *GRK1* and *SAG* [1]. Patients and especially male patients with the Schubert Bornschein-type of CSNB should be first screened in *CACNA1F* and *NYX* [1]. Both genes are located on the X-chromosome and represent the major causes of this form of CSNB. If a clinical discrimination of incomplete versus complete CSNB is made, only *CACNA1F* or *NYX* needs to be investigated. Our experience showed that at least 80% of these cases show mutations in one of those genes. Females and excluded male patients with icCSNB could be screened in *CABP4* and *CACNA2D4*, especially if they present with high hyperopia and photophobia. Cases of cCSNB should be screened for defects in *TRPM1* > *GRM6* > *GPR179* > *LRIT3*. In cases where no difference between icCSNB and cCSNB is made the following mutation detection strategy should be applied *CACNA1F* > *NYX* > *TRPM1* > *GRM6* > *GPR179* > *CABP4* > *LRIT3* > *CACNA2D4*. We developed this strategy, based on the prevalence of the specific gene defects [1]. Our recent experience showed that intronic variants and synonymous variants may be also disease causing and should not be overlooked [64]. In case only preliminary clinical phenotyping data are available unbiased microarray analysis (ASPER, Ophthalmics, Tartu, Estonia) [99, 100] and targeted next-generation sequencing (NGS) could be applied [101]. The prior method is based on allele-specific primer extension analysis, which allows the detection of known mutations. The array is regularly updated with new mutations in known genes and mutations that will be identified in novel gene defects. However, since there are only a few mutation hot spots and founder mutations in CSNB and their implicated genes, targeted NGS approaches seem to be more appropriate. Albeit, initially GC-rich and repetitive regions were less well covered by the latter methods, more recent techniques seem to overcome these challenges. After exclusion of mutations by the abovementioned method, targeted whole genome sequencing, whole exome or whole genome sequencing should be applied to identify the disease-causing mutation.

11.5 Summary

Inherited retinal disorders are very heterogeneous and can be deciphered depending on the congenital or progressive course of the disease or by the type of retinal cell that is involved. Herein we describe the genetic and phenotypic characterization of Congenital Stationary Night Blindness (CSNB). Depending on the mutated gene, CSNB patients can present a rod-photoreceptor defect (Riggs-type of CSNB) with or

without fundus abnormalities (Oguchi Disease, Fundus Albipunctatus) or a transmission defect from the photoreceptor to bipolar cells (Schubert-Bornschein type). The incomplete form of Schubert-Bornschein type of CSNB is due to a defect of proteins localized at the photoreceptor synapse while the complete form results from a ON-bipolar cell defect. Together with other clinical symptoms, clear genotype-phenotype correlations can be made as described herein.

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12 Genome Analysis for Inherited Retinal Disease: The State of the Art

Kate Oprych, Raquel Sofia Silva, Nikolas Pontikos, and Gavin Arno

Abstract

Inherited retinal disease (IRD) is a major global cause of blindness caused by mutations in a wide spectrum of genes essential to the retinal structure, maintenance and function. Current clinical diagnostic strategies in the UK are focused on targeted gene panel testing either by enrichment or virtually. Whole exome and genome sequencing (WES and WGS) have been used in rare disease genetic discovery now for a decade and are being integrated into many research pipelines and diagnostic strategies exemplified by the Genomics England 100,000 genomes project.

Here, we describe the current approaches to genetic and genomic analysis in IRD, the shortfalls and advantages of gene panel testing, WES and WGS in the context of single nucleotide, structural and copy number variants in coding, non-coding and intractable genomic regions.

Looking ahead, the missing heritability in IRD may be consequent on a number of factors: new genes, ignored or undetectable variants, new diseases for known genes, etc. Improved detection of genomic variation afforded by WGS paired with expanded variant databases, advances in variant interpretation, developing our understanding of the effect of non-coding variation using multiomics and integrating deep phenotyping and genomic data into machine learning tools will be the driving forces in better diagnosis of rare disease and discovery of novel causes of disease in the post-genomic era.

Keywords

Inherited retinal disease · Molecular genetics Whole exome sequencing · Whole genome sequencing · 100,000 genomes project Missing heritability · Multiomics · Artificial intelligence

K. Oprych
Great Ormond Street Hospital for Children, London, UK

R. S. Silva
UCL Institute of Ophthalmology, London, UK

N. Pontikos
UCL Institute of Ophthalmology, London, UK

Moorfields Eye Hospital, London, UK

G. Arno (✉)
Great Ormond Street Hospital for Children, London, UK

UCL Institute of Ophthalmology, London, UK

Moorfields Eye Hospital, London, UK
e-mail: g.arno@ucl.ac.uk

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12.1 Background

Inherited retinal disease (IRD) defines a broad spectrum of disorders characterised by retinal cell dysfunction and/or cell death, together representing a leading cause of visual impairment and blindness worldwide [1–3]. They affect an estimated 1 in 2000 individuals and over two million people globally [4].

The IRD spectrum of disease demonstrates vast phenotypic variability across multiple clinical parameters including the age of onset, severity and progression and can be broadly classified based on the primary cell type affected, rate of degeneration and whether the retinal disease occurs in isolation or with additional systemic features [4, 5]. Fundus imaging and functional testing with electroretinography are used for deep phenotyping and classification within IRD. The most common form of IRD is rod-cone dystrophy (or retinitis pigmentosa, (RP)) which has a prevalence of 1 in 4000 worldwide [6] while the commonest single-gene recessive disease is *ABCA4*-retinopathy with a carrier frequency estimated at 1 in 25–50 [7, 8] .

Retinal disease is a common presenting feature of a number of syndromic conditions including ciliopathies, lysosomal storage diseases and metabolic disorders: Usher's syndrome, Bardet Biedl syndrome (BBS), Senior-Loken syndrome and Joubert Syndrome being a few examples, and inheritance patterns for both non-syndromic and syndromic IRD can be autosomal dominant, recessive, X-linked and mitochondrial [4].

12.2 Genetics of IRD

Since the discovery of *RHO*, as the first gene is known to cause autosomal dominant RP by linkage and Sanger sequencing of gene candidates in 1990 [9], technological advancements have enabled the discovery of over 270 genes responsible for IRD (https://sph.uth.edu/retnet/) with many thousands of pathogenic and candidate variants now reported. There is indeed vast allelic and genetic heterogeneity within IRD making a hugely complex disease model. This can be exemplified by broad phenotypic variability consequent upon single genes and indeed single variants (eg: *CRX*) [10] and conversely many variants in many genes leading to an almost indistinguishable phenotype (eg: rod-cone dystrophy or RP). Some IRD genes also demonstrate incomplete penetrance, such as *PRPF31* which encodes precursor mRNA-processing factor 31, a ubiquitously expressed protein that is required for correct splicing of pre-mRNA transcripts [11]. Haploinsufficiency for *PRPF31* leads to symptomatic adRP in an estimated 50–60% of carriers and the rescue of the phenotype is thought to be consequent upon a second genetic determinant at the same locus on the trans allele [12]. A molecular diagnosis is important for more accurate risk predictions in these cases, where the inheritance pattern will be more challenging to recognise.

12.3 Genetic Screening Approaches

A molecular diagnosis provides many benefits to patients and families with rare disease; it enables the provision of more accurate information regarding risk predictions, prognosis and investigations, improved clinical care and management, access to treatments (current or emerging) and allows families to put a name to their genetic disease with associated non-clinical benefits.

Precise genotyping will become essential with the expansion of gene therapy in research and clinical settings [13, 14]. Of note, patients value having a molecular diagnosis and the option of predictive testing and are hopeful for emerging therapies for family members [15].

Current genetic screening available for IRD is territory dependant, in the UK this includes single-gene test Sanger sequencing, targeted gene panels and unbiased testing: whole exome and whole genome sequencing (WES and WGS). Deciding on the best option is directed by cost, setting (i.e. research or clinical setting) and confidence regarding the expected genotype. Where the phenotype is highly suggestive of a specific gene, such as *BEST1* [16], *CHM* [17], *TIMP3* [18], *EFEMP1* [19], *C1QTNF5* [20], Sanger

sequencing is still likely to be the initial approach to molecular diagnosis. However, in general, the vast heterogeneity of IRD means that consecutive sequencing of genes is an expensive, time-consuming and logistically challenging approach to establishing the molecular diagnosis [21]. Consequently, a comprehensive approach is required if pathogenic variants are not to be overlooked and for new genotype-phenotype correlations to be discovered.

The development of massively parallel sequencing, also known as next-generation sequencing (NGS) has revolutionised genetic discovery and molecular diagnostic testing for IRD and other Mendelian conditions [22–24]. NGS is a powerful high-throughput technology that can perform parallel sequencing of DNA on a vast scale and sequence an entire genome in a single experiment. Such techniques are now more frequently becoming the first-line diagnostic tool [21].

The spectrum of massively parallel sequencing platforms is broad, relying on a wide range of targeting chemistries, read length, sequencing technology and data processing. For example, DNA library preparation for massively parallel sequencing can be performed in a number of ways, including enrichment through polymerase chain reaction (PCR) amplification or capturing regions with DNA probes or the relatively unbiased PCR-free whole genome sequencing (WGS). For the purpose of this review, we will break down the methods based on this into three sub-groups: targeted gene panel testing, whole exome sequencing (WES) and WGS.

12.3.1 Targeted Gene Panels

In the UK, currently the commonest approach to IRD molecular testing is using an NGS-based targeted gene panel. Selected genes that are known to be associated with IRD, and increasingly, regions known to harbour pathogenic non-coding variants, are targeted for NGS using an enrichment step. One of the first of these for IRD was a 105-gene panel first trialled in RP cases [21]. Prior to this, routine access to genetic testing for IRD was limited in the UK to direct Sanger sequencing of single genes which was predominantly aimed at autosomal dominant RP and X-linked RP patients [21] or microarray analysis for known mutations (APEX array Asper Ophthalmics, Tartu, Estonia) [25]. The introduction of NGS gene panel testing was able to improve the diagnostic rates to approximately 80% in adRP cases and from 24% to 51% for broader IRD cohorts, demonstrating the advantage of this technology and the importance of wider access to genetic testing [21, 26]. Diagnostic rates were much higher in a paediatric cohort, with almost 80% of cases having a molecular diagnosis confirmed across all paediatric IRD [27]. Clinical assessment of children can be challenging due to limited cooperation and because the full phenotype may not yet be apparent, so early molecular diagnosis can facilitate the progress of children onto specific care pathways for screening and monitoring [27].

There are however several areas where targeted gene panels will miss diagnoses. Perhaps the greatest drawback of targeted panel testing is that they may be out of date often even before they can be properly implemented and continuously require updates as novel gene discoveries are made or important intronic variants in known IRD genes are identified [27, 28]. When panels are used in a clinical setting this requires extensive validation procedures which will limit the frequency of updates.

Furthermore, the ability of targeted enrichment to identify structural variants (SV) and copy number variants (CNV) is limited to read depth analysis for detection of alterations in dosage afforded by high coverage depth of targeted genes thus enabling effective detection of copy loss/gain [29]. Structural rearrangements are emerging as an important cause of IRD, accounting for significant proportions of disease alleles in recent studies [30, 31] and include variants not detectable by dosage analysis alone, for example, inversions, translocations and complex structural rearrangements with breakpoints rarely found in coding regions and thus may evade detection [28, 32]. Alternative genetic approaches such as CGH array and SNP array platforms may be used to augment the diagnostic pipeline and can improve diagnostic rates by an estimated 7% [33].

An argument against expanded analysis afforded by WES and WGS in the clinical setting is that diagnostic reporting of genetic variants must be rigorously validated. Therefore, clinical diagnostics must be focused on identifying 'provable' disease-associated variants, those being variants in known genes with a demonstrable protein-altering pathogenic mechanism be it the loss of function, damaging missense or splice altering variants. Although variants identified outside of the protein-coding regions, deep intronic and regulatory variants and novel gene associations are of great interest for the advancement of our understanding of the rare disease, they are difficult for clinical scientists to interpret. Therefore, the choice of genetic analysis for clinical diagnostics is a complex and carefully considered balance between the cost/benefit of applying WES/WGS versus gene panels.

12.3.2 Whole Exome Sequencing

Targeting of massively parallel sequencing to the exons of protein-coding genes, WES has become the widest used method for variant discovery studies in Mendelian disease in the research setting since the first gene discovery was reported a decade ago [22]. More recently, WES has been evaluated for use in the clinical diagnostic setting for IRD [31, 34–37]. With this technique, a 'virtual gene panel' may be applied targeting the analysis only on known IRD genes. It is predicted that more than 85% of disease-causing mutations are located in the exome [38]. Advantages of WES (and WGS) over panel-based testing is that disease-causing variants in off-panel genes are captured and as discoveries are made, reanalysis of unsolved patient cohorts is possible in light of new findings without the need to perform additional and costly experiments. Additional considerations with WES (and WGS) include the potential for identification of incidental findings; the American College of Medical Genetics (ACMG) has provided a recommended list of 59 highly penetrant genes that ought to be reported when WES and WGS are undertaken [39], although in the UK there is still debate about reporting of secondary findings [40–42].

WES has been reported to successfully identify the molecular cause of IRD in approximately 50–80% of cases in selected cohorts [34–37]. However, the coverage of known IRD genes by WES has been reported to be less effective than targeted panel tests [28, 43]. Consequently, clinical services currently prefer panel testing as the first-tier choice to IRD molecular diagnosis as it is cheaper, quicker, more sensitive and limits secondary findings [28, 44].

One of the main limitations of WES is that many regions of the genome known to harbour well characterised pathogenic variants exist outside of the coverage of WES enrichment kits. Therefore, it will not identify the increasing number of pathogenic non-coding variants accounting for a significant proportion of the missing heritability in IRD [31]. In addition, like targeted capture panels, enrichment is an integral part of the library prep methodology, thus leading to an artificially distributed coverage depth across the exome and challenges with read depth interpretation for SV/CNV detection. This is compounded by the fact that breakpoints in non-coding regions cannot be identified making confident calling difficult in many cases and validation more complicated, having to rely on qPCR, aCGH or MLPA in many cases.

12.3.3 Whole Genome Sequencing

WGS is the most comprehensive short-read sequencing technique for genome analysis, enabling interrogation of over 95% of the 3 billion nucleotide human genome. Superiority over other NGS technologies is evident from head-to-head comparison of coverage, diagnostic rates and by using WGS after other NGS methods have failed to identify causative variants [45–48], with Ellingford et al. [24] extrapolating a 29% improvement of WGS over targeted gene panels and Carss et al. [31] finding a 6% improvement of WGS over WES. The reasons for the

improvement in detection rates over other NGS techniques are multiple, including identification of pathogenic variants in non-coding regions, GC rich regions and structural variations.

The introns of genes associated with Mendelian disease may harbour pathogenic variants. Identifying pathogenic non-coding variants amongst all of the benign variations is inherently challenging: a frequent analogy being the 'needle in a haystack' due to the 3–4 million variants from the reference genome harboured in the average Illumina short-read sequencing genome. Therefore, accurate prediction of the effect of non-coding variants will prove key in delineating which variants are likely pathogenic [49].

Intronic variants are an important contributor to IRD causation through disruption of flanking splice sites and altering the strength of deeply intronic cryptic splice sites, resulting in cryptic splicing and protein disruption across many genes including *CHM, ABCA4, USH2A and CEP290* [24, 31, 50–55]. Causative intronic variants do account for significant proportions of unsolved cases with the CEP290 c.2991 + 1655A > G variant alone accounting for up to 21% of cases of LCA [56].

Furthermore, exonic variants may evade enrichment; GC rich regions are highly stable and therefore resistant to the denaturation stage of PCR resulting in poor coverage of these regions in certain WES applications. Carss et al. [31] demonstrated that in a patient with Leber congenital amaurosis, WGS identified heterozygous variants in exon 1 of *GUCY2D*, which has GC content of 76%, which at the time, WES would not have captured.

CNV and SV detection and characterisation using WES have inherent difficulties in that the breakpoints are often not covered at all meaning the only mechanism of detection available is based on the read depth [57]. Although effective, the lack of coverage uniformity that WES provides limiting and complex SV, inversions, translocations, etc. will be impossible to determine without a clear loss/gain. WGS on the other-hand enables incorporation of read depth analysis and split-read data analysis into bioinformatic pipelines which can detect complex structural variants and resolve breakpoints to the nucleotide level [31, 58, 59]. For example, in a patient with typical RP, WGS identified a structural variant in *EYS* which caused a 55 kb deletion (chr6: 65,602,819–65,658,187del) that encompassed exons 15–18, with both breakpoints deeply intronic, missed by WES [31]. With an estimated 5% of IRD patients harbouring a pathogenic SV/CNV, these variants are likely to account for a significant proportion of missing heritability by WES studies [31].

Finally, WGS also allows retrospective interrogation of the data as new IRD genes and pathogenic noncoding genes are discovered. Whilst targeted gene panels do include some non-coding variants the panels quickly become out of date. For example, Elllingford et al. [32] discovered intronic variants in *ABCA4* and *GPR98,* as well as a new IRD gene (*TRPM1*), when completing WGS in cases unsolved following a targeted gene panel because they were unknown at the time of panel design.

Areas that remain intractable to all NGS methods are those with highly repetitive regions and homologous pseudogenes due to the inevitable misalignment and mapping problems associated with short-read sequencing in these regions [31, 32]. The final exon of *RPGR* (ORF15) is a key example that highlights this issue and accounts for the majority of X-linked RP and hence, additional testing with optimised Sanger protocols is required to identify pathogenic variants for these cases [31, 60]. This is an issue that single molecule, long-read sequencing (aka third-generation sequencing) should resolve with read lengths of >20 kB enabling correct alignment and read through of repetitive sequences [61].

In the UK, clinical genetics is undergoing a revolution exemplified by the completion of the sequencing of 100,000 genomes from 70,000 individuals as part of the Genomics England 100,000 genomes project (100KGP). Funded by NHS-England, this study sequenced the genomes of some 3500 NHS patients with IRD [62]. Predominantly recruiting family trios (unaffected parents with affected offspring) in the rare

disease cohort provides unprecedented power for providing individuals with molecular diagnoses and making the discovery of new pathogenic variants and disease-associated genes, since *de novo* mutations in affected individuals, compound heterozygosity and homozygosity are readily apparent. Furthermore, it has launched the development and integration of WGS within a mainstream health service with the necessary infrastructure, education, research and industrial partnerships that are fundamental for NHS patients to benefit and for management of the vast amounts of data generated [63]. The cost-effectiveness of WGS over other NGS technologies for IRD and other disorders is not yet well characterised, and interpreting the health economics of these investigations is more complicated still [64]. However, with the expansion and development of the global genomic industry [65], the price of WGS continues to fall and WGS is likely to become the most cost-effective and comprehensive molecular diagnosis in IRD and similar conditions. Genomics England Interpretation Partnerships (GeCIP) have been established in this unique project to combine the expertise of researchers and clinicians to critically analyse the data from the 100KGP and embed research in clinical care [62, 66] with great promise for novel discoveries in IRD genetics [67–69].

12.4 Missing Heritability in Retinal Disease

Currently 40–60% [23, 24, 28, 31, 35, 37] of cases remain without a molecular diagnosis following NGS, depending on cohort differences and technologies used. There are a number of explanations that may accumulatively explain the missing heritability of IRD. Undetected variants in known IRD genes: many patients who undergo NGS testing are found to harbour a single disease allele in a compatible known recessive gene [31]. In such cases, it is likely that an unidentified variant in the same gene is present on the second allele. As discussed above, intronic variant and structural rearrangements including complex SV are emerging as important disease alleles, perhaps representing as high as 10–20% of mutations. In addition, variants in regulatory regions affecting promoters, enhancers and transcription factor binding sequences are further areas that remain difficult to elucidate. Several examples of regulatory region pathogenic variants have been well characterised to date in genes including *EYS, NMNAT1 and CHM* [70–72].

As researchers and clinical scientists employ less biased genetic strategies in the search for causative variants in Mendelian disease, the spectrum of pathogenic variants in syndromic disease genes becomes broader. There are many reports now in the literature of non-syndromic IRD cases with identified pathogenic or likely pathogenic variants in syndromic disease genes. This emerging phenomenon may represent the mild end of the syndromic disease spectrum with presumed hypomorphic alleles [31, 73–77] or novel associations, thought to represent different mechanisms of disease [78]. These examples highlight the importance of incorporating syndromic disease genes with a retinal component into targeted gene panels as well as virtual panels for WES/WGS in IRD testing strategies (https://panelapp.genomicsengland.co.uk/).

12.5 Multiomics

Now that whole genome analysis is quickly becoming the preferred tool of choice for identification of disease-causing variants for inherited disorders, it allows the identification of reported or novel variants that can affect gene expression, protein function, regulatory sequences or protein level, including by SV/CNV. However, the unbiased approach of reading an entire genome comes with a liability of overloading data.

Accomplishing the task of finding the causative variant(s), and potentially novel disease-causing genes, may be facilitated by the integration of information from different *omics* approaches, as well as patient phenotypic stratification and the reference population. The next layer of informa-

tion comes from the investigation of genomic, epigenetics and cellular mechanisms that sheds light on the interface of DNA-RNA-Protein dynamics.

The DNA molecule harbours a great deal of information beyond its linear sequence. In fact, genes account for only 2% of the pool of genomic material. The remaining 98% non-coding fraction is mainly made up of repetitive sequences that have a structural function in chromosome topology, but other parts are conserved across species and have regulatory activity. Cis-acting regulatory elements account for 6% of DNA, three times the equivalent of coding genes. But how does it all connect?

The 2 metre-long DNA molecule is found in the nucleus wrapped around octamers of 4 core protein histones that have amino acids tails that can be modified. The type of post-translational modification (e.g.: acetylation and methylation) and the amino acid location within the tail, impact directly on how compacted the DNA is in that particular stretch, and the different combinations, similarly to the nucleotides, work as a histone code. The linear gene sequence is composed of the promoter region, the gene body that accounts for the transcribed sequence, and the 5′ and 3′ untranslated regions (UTRs). Actively transcribed genes and regulatory sequences have open conformations and specific histone modifications (e.g.: Histone 3 Lysine 4 tri-methyl (H3K4me3), H3K27ac), which are associated with euchromatin, while repressed genes and elements are found tightly compacted and are decorated with histone marks associated with facultative heterochromatin (H3K27me3). A third state of chromatin, constitutive heterochromatin is associated with repetitive regions, including centromeric and telomeric regions of the chromosome, mainly supporting structural functions. These are decorated by silencing marks (such as H3K9me3 and H4K20me). Another level of information is the sequence position. DNA is organised in domains that are orderly insulated in the genome and preferentially interact with specific sequence regions. These are tightly associated with chromatin state. Furthermore, within the interacting domain, we find specific short or long-range cis-interacting elements, also known as enhancers. When active, these elements are bound by transcription activators or repressors that have a critical function in gene regulation. Promoter-enhancer interactions are often mediated by DNA looping [79] and are maintained by specific architectural factors and boundary insulator elements. The perturbation of this 3D structure can lead to gene mis-expression or ectopic expression [80, 81]. Therefore, faithful genome organisation and chromatin accessibility are key to ensure precise expression patterns. Pinpointing the exact molecular mechanisms driving genetic disease can be challenging, especially in the context of tissue development. The cell type can also exert a variance since the same gene could have different expression patterns and/or be regulated by different regulatory elements [82]. Assessing enhancer function and activity is hindered by the high level of enhancer redundancy [83].

Genome-wide profiling of chromatin immunoprecipitation sites for histone modifications and transcription factors, as well as DNA interacting domains by chromosome capture conformation, are fast being employed in the context of eye disorders and development [84–87]. Additionally, DNA methylation, DNA accessibility (including ATAC-seq), RNA sequencing of the different coding and non-coding transcripts, and proteomic assays, can further highlight genomic and genetic components that are preferentially important in the context of ocular development and disease [86, 88–91]. These approaches have contributed towards the identification of novel causative non-coding variants in eye disease [92–95].

A pertinent example of the employment of these NGS techniques is the discovery of the causative variants for North Carolina macular dystrophy (NCMD) and Progressive bifocal chorioretinal atrophy (PBCRA), two rare dominantly inherited disorders that affect central vision from birth [reviewed in reference 18]. Two linked loci had been identified at 5p21 and 6q16 [96–100], and in spite of many gene-sequencing approaches, no coding defect could be recognised. Moving the approach to genome-wide scale finally unrav-

elled the nature of the causal variants. So far 5 single nucleotide variants (SNVs) and 6 independent tandem duplications were identified on both loci. All 6 SNVs were found in two clusters in 6q, 15 kb and 7 kb upstream of the PRDM13 transcription start site, where both clusters were found located in DNase hypersensitive sites [92, 95]. The 3 tandem duplications at 6q also span these sites and include *PRDM13* sequence duplication [92, 101, 102]. In 5p21, three independent structural variants were identified with a combined shared region of 39 kb. This critical region for the phenotype is located in a gene desert downstream of *IRX1* and upstream of *ADAMTS16* [92, 94]. DNase-seq from human foetal retina also identified active sites at a restricted time during retinal development. Critically it was also proven that NCMD and PBCRA may represent a spectrum of the same disorder, dependent on the extent of dysregulation of the target genes affected by these regulatory variants. The most likely pathogenic mechanism is a gain of function, although it remains to be proven, due to constrains of modelling macula development.

Advances in sequencing technologies have also allowed the mainstream use of long-read sequencing, which has opened new views on novel RNA splicing variants and DNA structural variants complexity [103]. Additionally, the decrease in cost has spiked the use of single-cell technologies. Droplet-based single-cell sequencing was initially applied in adult mouse retina [104]. More recently it was applied in developing and adult primate retinas [105–107]. In the latest study, it was particularly used to compare primate-specific cell types such as foveal cells [106]. This has particular interest towards dissecting phenotypic aspects of eye disease, such as macular disorders.

Large scale studies adapting trio-based sequencing (GE 100KGP [62]; Deciphering Developmental Disorders, [108]), have to lead the way into personalised medicine. This approach allows significant reduction of candidate variants, and additionally, allows phasing of genomes and variants; *de novo* mutations and rare chromosomic phenomenon can also be readily identified. Combined these events are responsible for genetic diversity within the population and potentially within different tissues of an individual, since they can occur as germline mutations or in somatic tissue. This genetic diversity underlies human physiology and potentially accounts for both rare and common diseases. Furthermore, mosaicism may explain certain aspects of human disease such as penetrance and severity of the disorder [95, 109–111].

On certain occasions, even after the integration of all levels of information described above, the number of variants of unknown significance (VUS) can be substantial. VUS cannot be systematically tested individually and despite major improvements of *in silico* predictions [112], there is still a high level of inaccuracy, especially for the case of non-coding variants. High-throughput techniques have emerged with the potential to answer some of these questions. Saturation genome editing (SGE) resorts to the use of CRISPR-cas9 to create a library of hundreds of mutations that are tested in vitro and assayed in a single assay for a number of genes [113]. A similar mutagenesis based method was used to recreate 210 variants in Rhodopsin to test the effect on the expression of the protein [114]. As with any technique, the limitations of both these procedures are reliant on the metric used for inferring the causal consequence on gene expression.

The systematic combination of the different molecular approaches allows the identification of specific gene regulatory networks, adding instrumental power to achieving a personalised genomic analysis. Understanding the pathomechanism is fundamental for the patient and family members' prognostic, developing novel therapeutic strategies and selecting suitable participants for clinical trials. Non-coding variants present great potential for new pharmacological targets of intervention, since they avoid risk for off-target gene sequence alterations [115].

12.6 Imaging, Genetics and Artificial Intelligence for Inherited Retinal Disease Analysis

In the era of big data and global collaborations, computational methods are an essential part of rare disease genetic diagnosis and researchers in the IRD sphere are leading these advances. Computational tools have long been used for bioinformatics analysis for processing genetic data but now are also becoming part of phenotype analysis and clinical decision support. Combining approaches in genomic data interpretation with phenotype analysis tools will lead to better understanding through improved data integration, which coupled with artificial intelligence, will yield advances in genetic diagnosis and improved efficiency in clinical practice.

Next-generation sequencing technologies such as WGS offer us the most complete view of a human genome yet. However, as described above WGS detects hundreds of thousands of rare variants per individual posing a significant challenge in the interpretation of disease causality. Furthermore, many of these mutations occur in poorly characterised regions of the human genome.

Detailed phenotyping by experienced clinicians through careful patient interrogation and clinical tests, such as electroretinograms and detailed retinal imaging, can greatly aid the process of identifying the likely disease-causing mutations by identifying similarities with previously genetically diagnosed cases thus narrowing the search space for genetic mutations.

The description and definitions of phenotypes can vary widely between clinicians. This complicates meaningful comparisons across genetic cases and makes it harder to identify genes to phenotype correlations in IRD. This also makes computational analysis of phenotypes intractable. There have been many efforts to standardise phenotypes through the introduction of controlled vocabularies of clinical terms using encoding schemes such as SNOMEDCT and the UMLS [116]. For rare diseases such as IRD, the Human Phenotype Ontology (HPO) [117] is established as the favoured option and is now adopted by large projects such as the 100,000 genomes project led by Genomics England.

HPO terms are used in computational approaches to prioritise disease-causing variants and uncover novel gene to phenotype associations. Exomiser [118] prioritises variants based on phenotype similarity with published OMIM conditions, model organisms and gene pathways. Bevimed [119] uncovers gene to phenotype relationships based on phenotype similarity regressions using Bayesian statistics.

HPO descriptions are a first step towards enabling the integration of phenotype and genetic data to match patients with similar clinical features. Nonetheless annotating genetic cases with HPO terms still requires manual input which is difficult to fit into the already busy clinical workflow of large ophthalmic hospitals. Therefore, solutions are sought which facilitate the collection of HPO terms such as, making them part of the electronic health record entry system [120], by extracting these terms automatically from patient notes using natural language processing techniques, or even directly from imaging, ERGs or visual fields, are sought. A limitation of HPO terms is that they rely on subjective clinical terms. A more objective approach is to directly analyse the primary source of these HPO terms such as the imaging data.

Retinal imaging technologies are now widely and extensively used in ophthalmology due to modern advances in the field such as Optical Coherence Tomography (OCT), which allows detailed imaging of the layers of the retina, detection of oedemas, drusen and various other features symptomatic of retinal disease. Additionally, given the accessibility of the eye, retinal imaging is both very efficient and cost-effective and is now not only part of routine care at ophthalmic hospitals but also available to community opticians [121]. However, the interpretation of these images requires expertise acquired through years of training, and for IRD in particular, which are very rare and thus hard to recognise, these images

need to be inspected by clinical experts with in-depth knowledge of genetics, of which they are very few worldwide.

Such an IRD expert may be able to recognise the pattern of retinal deterioration which are gene specific and make prognosis as to the development of the disease [122, 123]. Nonetheless, this remains a subjective process dependent on the skills and experience of the clinician. There is also a shortage of such experts worldwide and this gap is increasing with the spread and accessibility to these new technologies. The gap is unlikely to close given that this knowledge takes years of experience to acquire.

Beyond the problem of shortage of experts, human-interpretation is also limited to recognising IRD genes by looking for known features. However there are also potentially new IRD gene-specific features in images which can only be discovered once we pool sufficient data.

The promise of Artificial Intelligence (AI) is to provide a more scalable, efficient and objective solution to IRD genetic diagnosis by training a neural network on retinal images of as many confirmed genetically diagnosed cases of IRD as possible. The trained AI could then suggest a gene given a retinal image from an IRD patient. This should lead to a more objective form of clinical diagnosis and has the potential of capturing the image pattern-recognition skills of the most advanced clinicians into a neural network and making them available as a clinical decision support tool. Along with aiding in finding the most likely genetic region affected which can help identify hard to detect genetic causes such as structural variants, non-coding mutations and silent mutations, this could also guide the clinician as to which genetic test is the most appropriate.

There are reasons to be optimistic that this approach can work, as AI has recently shown good results when applied to triaging eye scan from age-related macular degeneration and diabetic patients [124]. This approach has particularly illustrated the utility of a type of neural network, known as segmentation neural networks, are able to identify features in an image such as macular holes, odemas [125], drusens, and these can aid the quantitative analysis of these and link them to disease. These segmentations were then used as input to a second type of neural networks, known as classification neural networks. It is also possible to run a classification neural network known as convolutional neural network directly on the pixel intensities of images [126, 127]. However, the challenge is then to deconvolute the features that were used in predicting the outcome in order to explain the classification process.

A concrete proof-of-concept applied to IRD has recently been published by Fujinami-Yokokawa et al. in 2017 [128]. They trained a four-class CNN classifier to distinguish foveal OCT slices between three types of IRD patients with *RP1L1*, *EYS* and *ABCA4* retinopathies and healthy patients. The IRD patients had confirmed disease-causing mutations in these genes.

Nonetheless, these approaches are limited by the amount of training data which is why international data-sharing collaborations are particularly important to augment these training data-sets, especially for rare disease. One advantage of these AI approaches when applied to IRD over common disorders such age-related macular degeneration and diabetic retinopathy, is that the training labels are more reliable as they do not depend on subjective clinical interpretation, for example, wet vs dry, but instead on objective genetic data.

12.7 Conclusions

The continuous evolution of high-throughput sequencing technologies has critically advanced our knowledge on the human genome. This genomic revolution has enabled the incorporation of WGS into clinical diagnostic pipelines and led to the generation of unprecedented volumes of data, carrying associated implications in variant interpretation. The identification and characterisation of human genes and non-coding regulatory regions have revolutionised the field of human genetics and its application in the clinical setting by providing more efficient diagnostics and potential new pharmacological targets for intervention in a personalised fashion.

As NGS technology continues to develop, we will gain further insight into the role of genetic variation in human biology and disease, which will grant us a better understanding of the mechanism by which variants affect gene expression in the dynamic context of a cell, a tissue and the integration of all systems in the single organism. It is clear that as our ability to interpret genomic variation and the effect of non-coding variants improves, the advantages of WGS in the clinical realm will far outweigh its limitations perhaps leading to the replacement of targeted gene panels or WES as a clinical tool.

Indeed, as genetics and imaging become more accessible to the general public thanks to direct-to-consumer genetic testing, eye scanners used as part of eye-tests by community opticians, portable and home devices being developed, it is important to also democratise the interpretation knowledge of these complex data to avoid the risks and dangers of misinterpretation [129].

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13

Generation and Analysis of Induced Photoreceptor-Like Cells from Fibroblasts of Patients with Retinitis Pigmentosa

Yuko Seko

Abstract

Generation of induced photoreceptors (PRs) holds promise as a tool for in vitro modeling of inherited retinal diseases. Direct reprogramming, direct conversion or redirect differentiation of somatic cells by overexpression of transcription factors is a promising, simple, and low-cost approach to generate target cells from somatic cells without using induced pluripotent stem cells. My research group has successfully generated PR-like cells from human somatic cells; iris cells, dermal fibroblasts, and peripheral blood mononuclear cells (PBMCs) using this redirect differentiation technique. In this chapter, I introduce this method and demonstrate its application as a cellular model of inherited retinal diseases.

First, we tried to define the transcription factor combinations that can induce PR-like cells. A mixture of these genes was then transduced into iris cells, which were examined for inducible expression of PR-specific phenotypes. Expression patterns were dependent on combinations of transcription factors: A combination of *CRX* and *NeuroD* induced rhodopsin and blue opsin, but not green opsin; a combination of *CRX* and *RAX* induced blue opsin and green/red opsin, but not rhodopsin. After transduction with *CRX*, *RAX,* and *NeuroD*, rhodopsin-positive, blue opsin-positive, or green/red opsin-positive cells were found in induced PR-like cells by immunostaining, and these cells were determined to be photo-responsive by functional analysis using whole cell patch-clamp recordings. However, the response was an inward current instead of the typical outward current. Next, we tested whether human dermal fibroblasts could be converted into PRs. Transduction with the same combination of genes, *CRX*, *RAX,* and *NeuroD*, upregulated expression of the PR-specific genes. Additional *OTX2* gene transduction increased the upregulation of these genes. Both the *NRL* gene and the *NR2E3* gene were also endogenously upregulated in these cells. Global gene expression data by microarray analysis showed that phototransduction-related genes were significantly increased in these cells, where a photo-response, i.e., outward or inward current, was detected using the whole cell patch-clamp recordings. We then examined whether human PBMCs could be converted into PRs. Retinal disease-related genes, most of which are crucial to PR functions, were detected in *CRX*-transduced

Y. Seko (✉)
Department of Rehabilitation for Sensory Functions, Research Institute, National Rehabilitation Center for Persons with Disabilities,
Tokorozawa, Saitama, Japan
e-mail: seko-yuko@rehab.go.jp

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PBMCs. Functional studies showed that a light-induced inward current was detected in some *CRX*-transduced PBMCs.

Retinitis pigmentosa (RP) is an inherited retinal dystrophy that leads to visual impairment. The *EYS* gene was reported as the most common gene responsible for autosomal recessive (ar) RP. arRP with *EYS* gene defects is denoted by "EYS-RP." We produced PR-directed fibroblasts from EYS-RP patients using redirect differentiation as a replacement for degenerative retinas. A combination of four transcription factors, *CRX*, *RAX, OTX2,* and *NeuroD*, was transduced into dermal fibroblasts from three EYS-RP patients with homozygous or heterozygous mutations. We analyzed the defective transcripts of the *EYS* gene in these cells to elucidate the phenotypes of the EYS-RP patients, as the decay of the transcripts may be involved in the phenotypic variation associated with the disease. As a result, expression levels of defective transcripts were markedly different depending on the type of mutation. In conclusion, we suggest that the redirect differentiation method may be a valuable tool for disease modeling, despite some limitations.

Keywords

Redirect differentiation · Iris · Dermal fibroblast · Peripheral blood mononuclear cells (PBMC) · Photoreceptor (PR) · Disease modeling · Retinitis pigmentosa · *EYS* Truncating mutation · Nonsense-mediated mRNA decay (NMD) · Phenotypic variation Genotype-phenotype relationship

13.1 Introduction

Retinitis pigmentosa (RP) is an inherited retinal dystrophy that leads to visual impairment. Generation of induced photoreceptors (PRs) holds promise for in vitro modeling of inherited retinal diseases such as RP. The ideal tool for analysis of transcripts of the pathogenic genes is a retina from a patient, but for research purposes, cellular models are available as a substitute for human retinas. Induced PRs generated from disease-specific iPSCs of RP patients were reported to reproduce pathogenic phenotypes [1–4]. Although methods to generate PRs from induced pluripotent stem cells (iPSCs) have been established [5, 6], they are expensive and time-consuming. We established an alternative method, "redirect differentiation," wherein photosensitive PR-like cells are generated more easily and rapidly [7–9]. Direct reprogramming, direct conversion or redirect differentiation of somatic cells by overexpression of transcription factors is a promising, simple, low-cost approach to generate target cells from somatic cells without using iPSCs. My research group successfully generated PR-like cells from human somatic cells; iris cells, dermal fibroblasts, and peripheral blood mononuclear cells (PBMCs), using a redirect differentiation technique. Because we determined that continuous expression of exogenous transgenes is necessary to maintain the properties of PRs, we call this method "redirect differentiation."

We further generated and analyzed induced PR-like cells from human somatic cells derived from healthy volunteers and RP patients by redirect differentiation to examine the possibility of using these cells for disease modeling of RP.

13.2 What Is "Redirect Differentiation"?

The possibility of redirecting cell differentiation by overexpression of genes was first suggested by Weintraub with the identification of the "master gene," *MyoD* [10]. The process of "direct reprogamming" or "direct conversion" is thought to be direct lineage switching [11] rather than lineage switching back to a branch point and out again in a different direction. Examples of "direct conversion" has been shown in beta-cells, cardiomyocytes, and neurons: A specific combination of three transcription factors (Ngn3, Pdx1, and MafA) reprogram differentiated pancreatic exocrine cells in adult mice into cells that closely resemble beta-cells [12]; a combination of three

factors (Gata4, Tbx5, and Baf60c) induces non-cardiac mesoderm to differentiate directly into contractile cardiomyocytes [13]; and a combination of three factors (Ascl1, Brn2, and Myt1l) converts mouse fibroblasts into functional neurons [14]. We tried to generate PR-cells by this "direct reprogramming" or "direct conversion."

13.3 Methods of Differentiation and Assessment of Induced PR-Like Cells

At first, we defined the transcription factor combinations that can determine photoreceptor cell fate using human iris cells. Detailed methods are available in our previous paper [7]. In brief, we selected Six3, Pax6, Rax, Crx, Nrl, and NeuroD, genes that were expected to contribute to the induction of PR-specific phenotypes. Full length of transcription factors *SIX3* [15], *PAX6* [16], *RAX* [17], *CRX* [18], *NRL* [19, 20] and *NeuroD* [21], were amplified from cDNAs prepared from total RNA of adult human retina (Clontech, CA, USA) by PCR, and cloned into the XmnI-EcoRV sites of pENTR11 (Invitrogen). The resulting pENTR11-transcription factors were recombined with pMXs-DEST (modified pMXs (gift from T Kitamura to A Umezawa) by Y Miyagawa) by use of LR recombination reactions (Invitrogen). The retroviral DNAs were then transfected into 293FT cells and after 3 days, the media was collected and concentrated. The iris-derived cells were plated onto laminin-coated dishes and maintained for 1 day. The cells were transduced with media containing retroviral vector particles with 8 μg/ml of polybrene for 5 h at 37 °C. After retroviral transduction, the media was replaced with the DMEM/F12/B27 medium supplemented with 20 ng/ml bFGF, 40 ng/ml EGF, fibronectin, and 1% FBS. The retrovirus-transduced cells were cultured for up to 21 days. In order to measure the efficiency of transduction, we transduced retroviral eGFP under the same conditions. The frequency of eGFP-positive cells was 90–94% of all cells 48 h after transduction. Each vector contained one transcription factor and a mixture of vectors was used. Transduced cells were examined for inducible expression of PR-specific phenotypes using RT-PCR and immunocytochemistry. In addition, photo-responsiveness of induced PR-like cells was investigated using patch-clamp recordings.

13.4 Combinations of Transcription Factors Determining Photoreceptor Cell Fate

Transduction of a single gene for *SIX3*, *PAX6*, *RAX*, *CRX*, *NRL,* or *NeuroD* did not induce rod- or cone-specific phenotypes in iris cells, but the six genes together upregulated blue opsin and rhodopsin as shown previously [7]. To determine which of the six candidates were critical, we tested the effect of withdrawal of individual factors from the pool of transduced candidate genes on the expression of the opsin genes. We identified two genes, *NeuroD* and *CRX*, which were essential for PR-induction; withdrawal of *NeuroD* resulted in the loss of expression of rhodopsin, and withdrawal of *CRX* resulted in the loss of blue opsin. Expression patterns were dependent on combinations of transcription factors: A combination of *CRX* and *NeuroD* induced rod-specific genes, but did not induce the red opsin gene. Additional *RAX* gene transduction significantly upregulated blue opsin gene expression. A combination of *CRX* and *RAX* induced blue opsin and green/red opsin, but did not induce rhodopsin. *NeuroD* significantly decreased expression of the cone-specific genes, i.e., genes for green opsin and cone channel B3 (CNGB3) in human iris cells ($p < 0.005$). It was clearly demonstrated that the expression of rhodopsin and S-antigen, which are specifically expressed in rod-PRs, were much higher in *CRX*, *RAX,* and *NeuroD*-transduced cells than in *CRX* and *RAX*-transduced cells (rhodopsin, $p < 0.05$; S-antigen, $p < 0.005$, Welch's t-test). From these results, it was speculated that the combination of *CRX* and *RAX* generated immature PRs: and additional *NeuroD* promoted maturation.

We then tested whether human dermal fibroblasts could be converted into PRs [8]. Human

dermal fibroblasts can be differentiated to PR-like cells by the same transcription factor combination as human iris cells. Transduction of a combination of the *CRX*, *RAX,* and *NeuroD* genes upregulated expression of PR-specific genes, recoverin, blue opsin, and PDE6C. Additional *OTX2* gene transduction increased up-regulation of these genes. Both the *NRL* gene and the *NR2E3* gene, which were reported to determine photoreceptor cell fate, were endogenously upregulated in PR-directed fibroblasts by four transcription factors, *CRX*, *RAX, OTX2,* and *NeuroD*, by microarray analysis and endpoint RT-PCR, implying that exogenous *CRX*, *RAX, OTX2,* and *NeuroD*, but not *NRL*, are sufficient to generate PR-like cells with expression of rod-specific genes.

13.5 Endogenous and Exogenous Expression of Transcription Factors

We performed RT-PCR to investigate whether endogenous expression of transcription factors was induced in the PR-like cells that we generated [7, 8]. Both transgenic and endogenous *CRX, RAX,* and *NeuroD* were expressed. This indicates that human somatic cells, such as iris cells and dermal fibroblasts, were reprogrammed into PRs, at least to some extent. We then suppressed the *CRX* and *NeuroD* genes by siRNA. Expression of the PR-specific genes such as blue opsin, s-antigen, and recoverin decreased significantly in siCRX and siNeuroD-transfected cells, suggesting that continuous expression of *CRX* and *NeuroD* is necessary to maintain the properties of PRs. This is why we call our method "differentiation," not "reprogramming."

13.6 Photo-Responsiveness of Induced PR-Like Cells

For functional assessment of transduced cells, electrical recordings were made using the whole cell patch-clamp technique. The membrane current before and after light stimulation was recorded and analyzed. The PR-like cells derived from iris cells, induced by *CRX*, *RAX,* and *NeuroD*, responded to light. However, the response was an inward current instead of the typical outward current [7]. Since the light-induced inward current seemed to be mediated by melanopsin-associated phototransduction, we investigated the expression of melanopsin by RT-PCR and immunocytochemistry. *CRX*, *RX,* and *NEUROD*-transduced iris cells expressed melanopsin, suggesting that melanopsin expression was associated with inward current.

PR-directed fibroblasts, transduced by *CRX, RAX, NeuroD,* and *OTX2*, clearly responded to light. Global gene expression data by microarray analysis showed that phototransduction-related genes were significantly increased in induced PR-like cells. We also demonstrated that physiological responses to light differed between two different commercially available cell lines [22]. Under light stimulation, Ishii et al. found that an outward current (photoreceptor-like responses) was observed in both cell lines, while an inward current (intrinsically photosensitive retinal ganglion cell-like responses) was observed only in one cell line. Although cell age (passage number) may have differed, our data suggest that properties of the photosensitive cells produced by redirect differentiation may be controlled by the origin of the cell source.

However, some *CRX*-transduced PBMCs exhibited a light-induced inward current [9], instead of the typical outward current. Since the light-induced inward current seemed to be mediated by melanopsin-associated phototransduction as observed in iris-derived PR-like cells [7, 23], Komuta et al. investigated the expression of melanopsin by RT-PCR. The expression of melanopsin was not detected in PR-directed PBMCs. The reason an inward current was detected in *CRX*-transduced cells expressing PR-related genes was not determined; however, it might be possible that signals passed from blue, red, or green opsin to a downstream point in the melanopsin signaling cascade in *CRX*-transduced cells, leading to depolarization by light stimulation.

13.7 Variation of Cell Types of Sources for Induced PR-Like Cells (Fig. 13.1)

13.7.1 Iris

My research group defined the transcription factor combinations that can induce PR-like cells from human infantile iris cells [7]. Expression patterns were dependent on combinations of transcription factors: A combination of *CRX* and *NeuroD* induced rhodopsin and blue opsin, but not green opsin; a combination of *CRX* and *RAX* induced blue opsin and green/red opsin, but not rhodopsin. Expression levels of rhodopsin genes and blue opsin genes reached maximum levels 1 week after gene transduction of transcription factors and remained unchanged for up to 3 weeks. Expression of green/red opsin reached maximum levels 3 days after gene transduction. After transduction with *CRX*, *RAX,* and *NeuroD*, rhodopsin-positive, blue opsin-positive, and green/red opsin-positive cells were 29% (per 954 cells), 37% (per 235 cells), and 25% (per 193 cells) of total cells, respectively, by immunostaining. Hybrid PRs were also detected by double-staining immunocytochemistry. Ultrastructural analysis revealed a cilia-associated structure, i.e., centriole, surrounded by mitochondria [7].

Although it has been shown that retinal stem cells are not present in the human iris [24, 25], our previous study demonstrated that human iris cells expressed stem cell markers such as nestin, N-cadherin, Sox2, Musashi-1, and Pax6 [7]. Expression of stem cell markers in iris cells may be attributed to the cell source, i.e., cells from infants. However, PR cell differentiation with exogenously added chemicals and growth factors was limited [7]. Other experimental evidence has also suggested the limitation in mammals without genetic manipulation. Progenitor cells from the mammalian iris, pars plana, and ciliary body do not show a convincing immunoreactivity for rhodopsin, phosducin, recoverin, PKC, or RPE65 [26], but are induced into PR progeny with retinal transcription factors [27, 28]. Derivation of PR-like cells can be attributed to transgene-dependent differentiation of retinal progenitors that exist in the iris. We also indicated that human iris stromal (IS) cells that originate from neural crest [7], as well as IPE cells, differentiate into PR-like cells.

13.7.2 Dermal Fibroblasts

The induced pluripotent stem cells (iPS) developed by Takahashi and Yamanaka was the first

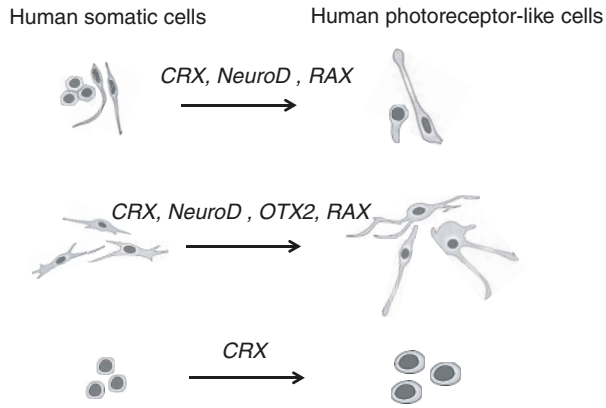

Fig. 13.1 Variation of cell types of sources for induced photoreceptor-like cells

Cell source	Characteristics	Origin	Invasiveness	Reference
Iris cells	attached	eye	high	Seko et al. 2012 (7)
fibroblasts	attached	dermis	medium	Seko et al. 2012 (8)
mononuclear cells	floating	blood	low	Komuta et al. 2016 (9)

model for "direct reprogramming," in which mouse adult fibroblasts were reprogrammed by transduction of four transcription factor genes, Oct3/4, Sox2, c-Myc, and Klf4 [29]. Additionally, functional neurons were generated from mouse fibroblasts by a combination of three factors (Ascl1, Brn2, and Myt1l) [14], and functional platelets were generated from mouse and human fibroblasts by a combination of three factors (p45NF-E2, MafG, and MafK) [30]. Because human dermal fibroblasts are less specialized than iris cells, we tested whether human dermal fibroblasts could be converted into PRs by the same defined combination of genes used successfully for human iris cells, *CRX, RAX,* and *NeuroD*, to generalize and establish our technology for generating PRs [8]. In human dermal fibroblasts, recoverin, blue opsin, PDE6c were upregulated by a combination of *CRX, RAX,* and *NeuroD*. Additional *OTX2* gene transduction increased up-regulation of the PR-specific genes; that is blue opsin, recoverin, S-antigen, CNGB3, and PDE6C. These results suggest that *OTX2* may work as an amplifier [8].

For functional assessment of transduced cells, electrical recordings were made using the whole cell patch-clamp technique. The membrane current before and after light stimulation was recorded and analyzed. Induced PR-like cells derived from human dermal fibroblasts, induced by *CRX*, *RAX,* and *NeuroD*, responded to light. A typical outward current was detected [8, 22].

Dermal fibroblasts are of mesodermal origin and immunogenic, while iris-pigmented epithelial cells (IPE cells) are of neural ectoderm origin and show immune tolerance. Iris cells studied here include not only IPE cells but also iris stromal cells, which are of neural crest origin. We have previously shown that iris cells, IPE cells, and iris stromal cells are differentiated into photoreceptor cells in the same way [7]. However, dermal fibroblasts are harvested easily and safely, and iris cells are obtained surgically. To find a more suitable cell source than the iris cells for reprogramming into photoreceptor cells, we compared signal ratios between PR-direct fibroblasts and PR-directed iris cells by a microarray analysis. The results show that there was an increase in both the expression levels and the variety of upregulated PR-specific genes in PR-directed iris cells when compared with PR-direct fibroblasts [8]. The difference in induced endogenous expression of transcription factors *CRX, RAX,* and *NeuroD* between CRN-Fib and CRN-Iris as well as the difference in upregulated photoreceptor-specific genes may suggest a difference in reprogramming potential between the two types of cells. From the standpoint of regenerative medicine, iris cells may be more suitable than dermal fibroblasts based on their characteristics of immune tolerance and higher expression of retina-specific genes in differentiated cells. It may be possible to improve dermal fibroblasts as a source by use of other transcription factors or by manipulating the histone methylation signature [31]. Dermal fibroblasts have an important advantage in that these cells are obtained safely and easily from patients. Because the direct reprogramming method may be suitable to provide the small numbers of cells required for individualized drug screening and disease modeling, dermal fibroblasts may be useful for such purposes despite their limitations.

13.8 Peripheral Blood Mononuclear Cells (PBMCs)

We further investigated another cell type, peripheral blood mononuclear cells, or PBMCs. Though dermal fibroblasts are often utilized for reprogramming, sampling by dermal biopsies requires surgical intervention and expertise. Therefore, we tested whether human PBMCs could be converted into PRs. Based on our previous studies of the generation of photosensitive PR-like cells from human iris cells and human dermal fibroblasts, we transduced the same transcription factors into PBMCs via Sendai virus vectors. PBMCs expressed cone-related genes after the transduction of *CRX* alone using SeV vectors. Blue opsin and red/green opsin were more efficiently and intensely expressed in

CRX-transduced PBMCs prepared using SeV vectors than in those using retrovirus vectors because transduction by SeV vectors is efficient. However, the expression levels of the blue opsin gene increased in *CRX*-transduced PBMCs but not in fibroblasts, although transduction was performed by SeV vectors in both cell types. Endogenous *CRX* expression was detected in dermal fibroblasts transduced with *CRX* by both retrovirus and SeV vectors, but was detected in PBMCs transduced only by SeV vectors. These differences might be attributed to variable reprogramming efficiencies based on different methylation signatures dependent on cell types, as previously reported [31, 32].

We found that some PR-related genes, blue opsin, PDE6H, and SAG, were efficiently detected in *CRX*-transduced cells. Expression levels of blue opsin and PDE6H peaked at 1 week and that of SAG peaked earlier, at 3 days. By immunocytochemistry, on the third day after transduction, blue opsin-positive cells constituted about 20% of the CRX-transduced cells. Surprisingly, in functional studies, patch-clamp recordings showed that a light-induced inward current was detected in some *CRX*-transduced cells. Photostimulation of the rod or cone pathway produces hyperpolarizing responses, while activation of the melanopsin pathway produces depolarizing responses [23]. Since the light-induced inward current seemed to be mediated by melanopsin-associated phototransduction as observed in iris-derived photoreceptor-like cells [7], Komuta et al. investigated the expression of melanopsin by RT-PCR. However, the expression of melanopsin was not detected in photoreceptor-directed PBMCs. We therefore examined photoreceptor-related and melanopsin-related genes that function in phototransduction. Strong expression of downstream genes of the melanopsin cascade, such as TRPC and Gqα, was detected. Abundant *CNGB3* expression was detected in *CRX*-transduced cells, but *CNGA3*, which coordinates with CNGB3, was not sufficiently expressed. This might be the reason why the phototransduction cascade could not mediate the light stimuli as the typical outward current of photoreceptors. Proteins involved in the signal transduction cascade of melanopsin, such as TRPC and Gqα proteins, which induce depolarization, were abundantly expressed, while Gαt and CNG proteins, which induce hyperpolarization, were not sufficiently expressed. The reason why an inward current was detected in *CRX*-transduced cells expressing photoreceptor-related genes was unknown at this time; however, it might be possible that signals passed from blue, red, or green opsin to a downstream point in the melanopsin signaling cascade in *CRX*-transduced cells, leading to producing the depolarization by light stimuli.

Furthermore, numerous retinal disease-related genes were efficiently detected in *CRX*-transduced cells, most of which are crucial to the photoreceptor function. In order to increase differentiation efficiency, Komuta et al. modified the culture conditions. By adding transduction of *RAX1* and *NEUROD1*, additional conditioned medium of cultured retinal pigment epithelial cells and Activin A, DKK, and Lefty2, they saw expression of a greater variety of retinal disease-related genes than that observed in *CRX*-transduced PBMCs. Polycistronic vectors, with four transcription factors, *CRX, RAX*, *NeuroD,* and *OTX2*, were inserted in a cistronic manner via Sendai virus, were employed with the aim to improve the differentiation efficiency of PBMC to PRs. However, expression levels of rhodopsin were higher in PR-directed fibroblasts with a mixture of mono-cistronic retrovirus vectors than in PR-directed PBMC by polycistronic vectors via Sendai virus vectors (unpublished data).

PBMC proliferation is induced by IL-2, are easily collected, and are safer to use compared to dermal fibroblasts; these cells have the potential for use as a cell source for differentiation into PRs. *CRX* transduced by SeV acts as a master control gene for reprogramming of PBMCs into PRs, specifically, cone PR-like cells. In PR-directed PBMCs, expression of rod-photoreceptor specific genes was very low; the differentiation needs to be improved.

13.9 Application of Induced PR-Like Cells to RP Research

We examined the possibility of using our induced PR-like cells derived from dermal fibroblasts of RP patients as disease modeling for RP [33] because of the shortcomings of our differentiation methods.

RP displays degeneration of PR/RPE via gene defects, leading to the deterioration of nyctalopia and narrowing of the visual field. RP is progressive and incurable, leading to a major causative disease of juvenile blindness. It is speculated that gene defects lead to cellular dysfunction of PRs in patients. Disease modeling of RP should be useful for disease diagnosis, elucidation of pathogenesis, and drug screening.

Defects in the *EYS* gene on chromosome 6q12 were found to be a major cause of autosomal recessive (ar) RP in several populations [34–39]. In Japan, c.4957dupA (p.Ser1653Lysfs*2) and c.8805C > A (p.Tyr2935*) were identified as pathogenic mutations in about 20%–30% of arRP patients [40, 41]. To date, many EYS variants have been reported as causative defects of RP [42]. Hereafter arRP caused by defects in the *EYS* gene is denoted as "EYS-RP." RP is a highly heterogeneous disease, and accordingly, EYS-RP exhibits heterogeneous phenotypes with a wide range in severity. In order to clarify the genotype-phenotype correlation in EYS-RP, the analysis of transcripts may be helpful. *EYS* (OMIM 612424) is currently the largest gene expressed in the human eye, spanning over 2 Mb within the RP25 locus (6q12) [34, 37]. The ideal tool for analysis of the *EYS* gene transcripts is a retina from an EYS-RP patient. For research purposes, cellular models are available as an alternative for human retinas.

We collected fibroblasts of patients with "EYS-RP." Dermal fibroblasts were harvested from three healthy donors: N#1, N#2, N#3, and three EYS-RP patients with homozygous or heterozygous mutations (Table 13.1) under the approval of the Ethics Committee of the National Rehabilitation Center for Persons with Disabilities (NRCPD). Using "redirect differentiation" by *CRX*, *RAX*, *NeuroD,* and *OTX2*, we generated PR-directed fibroblasts derived from these subjects. We tested the inducible expression of the PR-specific genes (blue opsin, rhodopsin, recoverin, S-antigen, PDE6C, EYS) in these cells. PR-specific genes were upregulated in all the PR-directed fibroblasts tested. However, expression levels of defective transcripts of the *EYS* gene were markedly different, depending on the type of mutation. To analyze transcripts derived from three different types of the defective *EYS* gene, c.1211dupA, c.4957dupA, and c.8805C > A, we performed RT-PCR and analyzed DNA sequences of amplified products for exon 6–11, exon 26–27, and exon 42–43 that carry c.1211dupA, c.4957dupA and c.8805C > A, respectively, using total RNAs extracted from PR-directed fibroblasts of Pt#1, Pt#2 and Pt#3 10 days after gene transduction. Transcripts derived from these three defective genes were barely detectable, expressed at a lower level, or expressed at almost the same level as in normal volunteers, respectively.

Table 13.1 Defects in the *EYS* gene in patients

Patient #	Allele 1	Allele 2
	Mutation	Mutation
1	c.4957dupA	c.4957dupA
2	c.4957dupA	c.8805C > A
3	c.4957dupA	c.1211dupA

Generally, faulty transcripts are immediately triaged for destruction by nonsense-mediated mRNA decay (NMD) [43]. All three EYS-RP donors had the frameshift mutation c.4957dupA, in at least one allele of exon 26 (Table 13.1). Therefore, we expected that NMD would cause the loss of the *EYS* gene transcripts corresponding to exon 26–27. However, the transcripts with c.4957dupA were clearly detected in PR-directed fibroblasts from Pt#1, carrying homozygous mutations and Pt#2, carrying compound heterozygous mutations. However, the expression levels of the transcript with c.4957dupA in Pt#1 and Pt#2 were lower than the mean of N#1, N#2, and N#3. To explain this phenomenon, we referred to a previous study [44] where it was reported that a *cis* element that inhibits NMD is located within the first 200 nt when positioned in the down-

stream proximal region of the premature termination codon (PTC) [44]. The authors showed several examples with significant enrichment of A/U nucleotides (63%–71%) and hypothesized that this may be a condition for NMD evasion. To determine whether our data was consistent with their hypothesis, we analyzed sequences in the proximal downstream region of the PTC in the transcripts derived from defected alleles of the *EYS* gene and calculated the A/U nucleotide content. For c.4957dupA, A/U content was 67%, which is in the range previously reported (63%–71%). This result supports our hypothesis that transcripts having the frameshift mutation, c.4957dupA, may partially escape from NMD.

Pt#2 has the nonsense mutation c.8805C > A, on an allele of exon 43 (Table 13.1). Because this mutation produces a PTC in the last exon, the transcript with this mutation may escape degradation by NMD [45]. These transcripts, corresponding to exon 42–43, were clearly detected in PR-directed dermal fibroblasts derived from Pt#2. The peak amplitudes of normal and mutated bases on the electropherogram were nearly the same. The expression levels from Pt#2 were similar to those from normal volunteers, suggesting that escape from NMD occurred in transcripts with c.8805C > A. Interestingly, the exon 42–43 region of the *EYS* gene was expressed in human dermal fibroblasts without PR-induction. The expression level of the exon 42–43 fragment in default state fibroblasts was higher than in PR-directed fibroblasts. Because the EYS is reported to be expressed exclusively in the retina, our research group intensively studied on this exon 42–43 fragment that is expressed in human dermal fibroblasts. As a result, Takita et al. identified a new variant, transcribed from exon 37, which is specifically expressed in dermal fibroblasts [46].

Pt#3 has the frameshift mutation c.1211dupA (p.Asn404Lysfs*3) in exon 8, which has previously been reported in an Israeli arRP patient [38]. By endpoint RT-PCR and sequencing, only the transcript derived from the normal allele was detected, suggesting that the transcript derived from the mutant allele was degraded by NMD, as expected.

To pursue the relationship between phenotypic variations of EYS-RP patients, large samples are needed. The present study also suggests that the redirect differentiation method could be a valuable tool for disease modeling, despite some limitations. Our induced PR-like cells may contribute to individualized drug screening and disease modeling of inherited retinal degeneration.

13.10 Conclusion

My research group has successfully generated PR -like cells from human somatic cells; iris cells, dermal fibroblasts, and peripheral blood mononuclear cells (PBMCs) using a redirect differentiation technique. Expression patterns of PR-specific genes were dependent on combinations of transcription factors in PR-like cells that we generated.

By the redirect differentiation technique, an in vitro EYS-RP model was created by transduction of a combination of transcription factor genes, *CRX, RAX, NeuroD, and OTX2*, into dermal fibroblasts derived from EYS-RP patients with homozygous or heterozygous mutations. The expression of the defective *EYS* transcripts was markedly different, depending on the type of mutation. Nonsense mutations of the *EYS* gene transcripts, which are the same as in the genome, were detected. These results suggest that nonsense-mediated mRNA decay, NMD, is inhibited, in part, by a cis-acting mechanism. Molecular changes in the in vitro model of RP mimic the pathological condition of RP, in part.

In conclusion, we believe that our redirect differentiation method may be a valuable tool for disease modeling, despite some limitations.

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14

Genotype–Phenotype of *RPE65* Mutations: A Reference Guide for Gene Testing and Its Clinical Application

Zhen Yi, Christina Zeitz, Takeshi Iwata, J. Fielding Hejtmancik, and Qingjiong Zhang

Abstract

The *RPE65* gene encodes a retinal pigment epithelium-specific isomerase that catalyzes the conversion of all-trans retinyl esters to 11-cis retinol, the activity of which affects the formation of visual pigment in photoreceptors. Mutations in *RPE65* in inherited retinal dystrophies have been studied widely worldwide, especially now that gene therapy for patients with *RPE65* mutations is available in the clinic. The aim of this study is to reveal the *RPE65* mutation spectrum and frequency, the associated phenotypic characteristics, and potential genotype–phenotype correlations. In total, 201 mutations in *RPE65* were identified in 479 patients from 353 families based on data reported in the literature. Mutations in 349 families caused autosomal recessive retinal degeneration, while a c.1430A>G (p.Asp477Gly) mutation in four families resulted in autosomal dominant retinal degeneration resembling retinitis pigmentosa, choroideremia, or vitelliform macular dystrophy with incomplete penetrance. Mutations identified in families with biallelic *RPE65* mutations included missense (113/200 [56.5%]), frameshift indel (39/200 [19.5%]), splicing defect (24/200 [12.0%]), nonsense (19/200 [9.5%]), inframe indel (4/200 [2.0%]), and start loss (1/200 [0.5%]). A significant reduction in visual acuity was noticeable at 15 years of age and at 35 years of age, suggesting a crit-

Z. Yi
State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China

C. Zeitz
Sorbonne Université, INSERM, CNRS, Paris, France
e-mail: christina.zeitz@inserm.fr

T. Iwata
Division of Molecular and Cellular Biology, National Institute of Sensory Organs, National Tokyo Medical Center, Tokyo, Japan

Global Eye Genetics Consortium (GEGC), Tokyo, Japan
e-mail: takeshi.iwata@kankakuki.go.jp

J. F. Hejtmancik
Ophthalmic Genetics and Visual Function Branch, National Eye Institute , National Institutes of Health, Bethesda, MD, USA

Global Eye Genetics Consortium (GEGC), Tokyo, Japan
e-mail: hejtmancikj@nei.nih.gov

Q. Zhang (✉)
State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China

Global Eye Genetics Consortium (GEGC), Tokyo, Japan
e-mail: zhangqji@mail.sysu.edu.cn; zhangqingjiong@gzzoc.com

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ical window for treatment including gene therapy. Two major types of fundus changes were observed: (1) mild or obvious tapetoretinal degeneration, generalized or located mainly in the mid-peripheral retina; and (2) fundus albipunctatus-like retinopathy. Typical bone-spicule pigmentation was rarely seen in early childhood but may be observed after 35 years of age. A severe phenotype (Leber congenital amaurosis) is frequently associated with biallelic loss-of-function mutations, while milder phenotypes are more likely to be associated with one or two missense mutations. The overall information presented here should be useful as a reference guide in clinical practice, especially for clinical gene testing and enrollment in gene therapy.

Keywords

RPE65 · Mutation spectrum and frequency Genotype–phenotype · Inherited retinal dystrophies · Leber congenital amaurosis

14.1 Introduction

RPE65 is a 65-kDa protein specific to the retinal pigment epithelium (RPE) [1], which is a single cell-layered tissue in close contact with the photoreceptor outer segments. The *RPE65* (MIM: 180069) gene maps to chromosome 1p31.3 and contains 14 exons, encompassing 21 kb of genomic DNA. It encodes the isomerase that catalyzes the conversion of all-trans retinyl esters to 11-cis retinol in the RPE, an essential step in the metabolism of vitamin A [2]. Without sufficient activity of this gene product, no active chromophore can be produced.

Mutations in *RPE65* have been reported in patients with a variety of autosomal recessive inherited retinal dystrophies (IRDs), including Leber congenital amaurosis (LCA [MIM: 204100]), early-onset severe retinal degeneration (EOSRD), and retinitis pigmentosa (also called rod–cone dystrophy, RP [MIM: 268000]). In addition, nine patients with a phenotype resembling fundus albipunctatus (FA [MIM: 136880]) have been described [3–5]. A heterozygous *RPE65* mutation, c.1430A>G (p.Asp477Gly), was reported to cause autosomal dominant retinal degeneration of varied phenotypes in four families with incomplete penetrance [6, 7]. LCA is the earliest and most severe form of IRDs. Symptoms and signs of LCA usually appear in the first year of life and include nystagmus, oculodigital signs, a sluggish pupillary light reflex, retinal degeneration, and severely reduced or extinguished electroretinography [8]. LCA is both clinically and genetically heterogeneous. EOSRD is a subgroup of retinal dystrophies between LCA and RP and was first described by Leber in 1916, with symptoms appearing in early childhood after 1-year old and often leading to blindness by age of 30 years [9]. RP is the most common form of IRDs, with signs and symptoms appearing in juveniles or adults. It is characterized by night blindness, constriction of the visual field, gradual reduction in visual acuity, waxy pale optic discs, attenuated retinal arteries, and pigmentary abnormality initially located in the mid-peripheral retina [10]. There is a considerable clinical overlap among LCA, EOSRD, and RP. In some reports, the diagnosis is ambiguous. FA, a group of relatively stationary diseases, mainly manifests as night blindness with myriad round white or white-yellow dots in the mid-periphery fundus [3, 4].

In recent years, due to the feasibility of gene therapy in the clinic [11], research on *RPE65* has intensified. Previous reviews on *RPE65* mainly focused on gene therapy trials and mutations in LCA patients [12–14], rather than reviewing all mutations and the relationship between mutations and phenotypes. In this study, we provide an overview of all mutations of *RPE65* in different forms of IRDs, mainly in patients with biallelic *RPE65* mutations reported thus far, as well as their associated phenotypes. Based on the available phenotypic data, we summarize the phenotypic characteristics of patients with *RPE65* mutations. In addition, we analyze potential correlations between *RPE65* genotypes and the clinical features of patients, which might assist in clinical gene testing, time win-

dow for gene therapy, and possible prognosis of the disease.

14.2 Methods

The keyword "RPE65" was searched on PubMed (https://www.ncbi.nlm.nih.gov/pubmed/), Web of Science (http://apps.webofknowledge.com/WOS_GeneralSearch_input.do?product=WOS&SID=8BApKgHrMnA4WVjh45d&search_mode=GeneralSearch), and Google Scholar (http://so.hiqq.com.cn/) on February 22, 2019. All available literature reporting *RPE65* mutations and corresponding phenotypic data published in English was collected. The number of different mutations, the frequency of each mutation, and the potential genotype–phenotype correlation were summarized.

14.3 Results

14.3.1 Number of Publications and Patients

A total of 479 patients from 353 families with *RPE65* mutations have been reported in 111 publications to date [3–7, 9, 15–119]. This group comprises 472 patients from 349 families with biallelic *RPE65* mutations reported in 109 publications and only 7 patients from 4 families with heterozygous *RPE65* mutations reported in 2 publications. Due to the small number of heterozygous families, we mainly analyzed the genotype–phenotype correlation of patients with biallelic *RPE65* mutations herein.

14.3.2 *RPE65* Mutational Profile

To date, only one heterozygous mutation (c.1430A>G, p.Asp477Gly) in *RPE65* has been identified in seven patients from four families with autosomal dominant retinal degeneration. In contrast, 200 biallelic mutations in *RPE65* were identified in 698 chromosomes of 349 families with autosomal recessive retinal degeneration. These 200 mutations can be classified as missense (113/200 [56.5%]), frameshift indel (39/200 [19.5%]), splicing defect (24/200 [12.0%]), nonsense (19/200 [9.5%]), inframe indel (4/200 [2.0%]), and start loss (1/200 [0.5%]) (Fig. 14.1a). The combination of the different biallelic mutations can be grouped into 15 categories, with the combination of a patient having two heterozygous missense mutations ("missense + missense") being the most common (161/349 [46.1%]) (Fig. 14.1b). On the basis of the 200 mutations and genotypes of 349 families, the frequency of each mutation in 698 mutant alleles was summarized and shown in the mRNA sequence (Fig. 14.1c). The four most common mutations affecting amino acid residues Arg91 and Tyr368 and splice defect mutations in intron 1 and intron 2 account for only 26.8% (187/698) of all mutant alleles, suggesting an absence of mutation hot spots in this gene. This is further supported by an even distribution of the 200 mutations on all 14 exons (Fig. 14.1c).

14.3.3 Visual Acuity of Patients with *RPE65* Mutations Decreases with Age

Reviewing the available best visual acuity (VA) and age, we obtained a total of 261 VA measurements collected from 227 patients (Fig. 14.2a). Each patient had a varying number of measurements (minimum, 1; maximum, 4). The age of the patients contributing VA measurements to the analysis ranged from 0.3 to 61 years. Among the 261 VA measurements, 38.7% were lower than 0.05 Snellen equivalent (legal blindness), and 42.9% were lower than 0.3 but higher than 0.05 (low vision). Among cases of legal blindness (lower than 0.05), no light perception (NLP) accounted for 5.9%, light perception (LP) accounted for 30.7%, hand motions (HM) accounted for 16.8%, count finger (CF) accounted for 15.8%, and measurable VA accounted for 30.7%. There was a clear trend toward lower values with increasing age. Most patients 35 years old showed worse visual acuity with a clustering

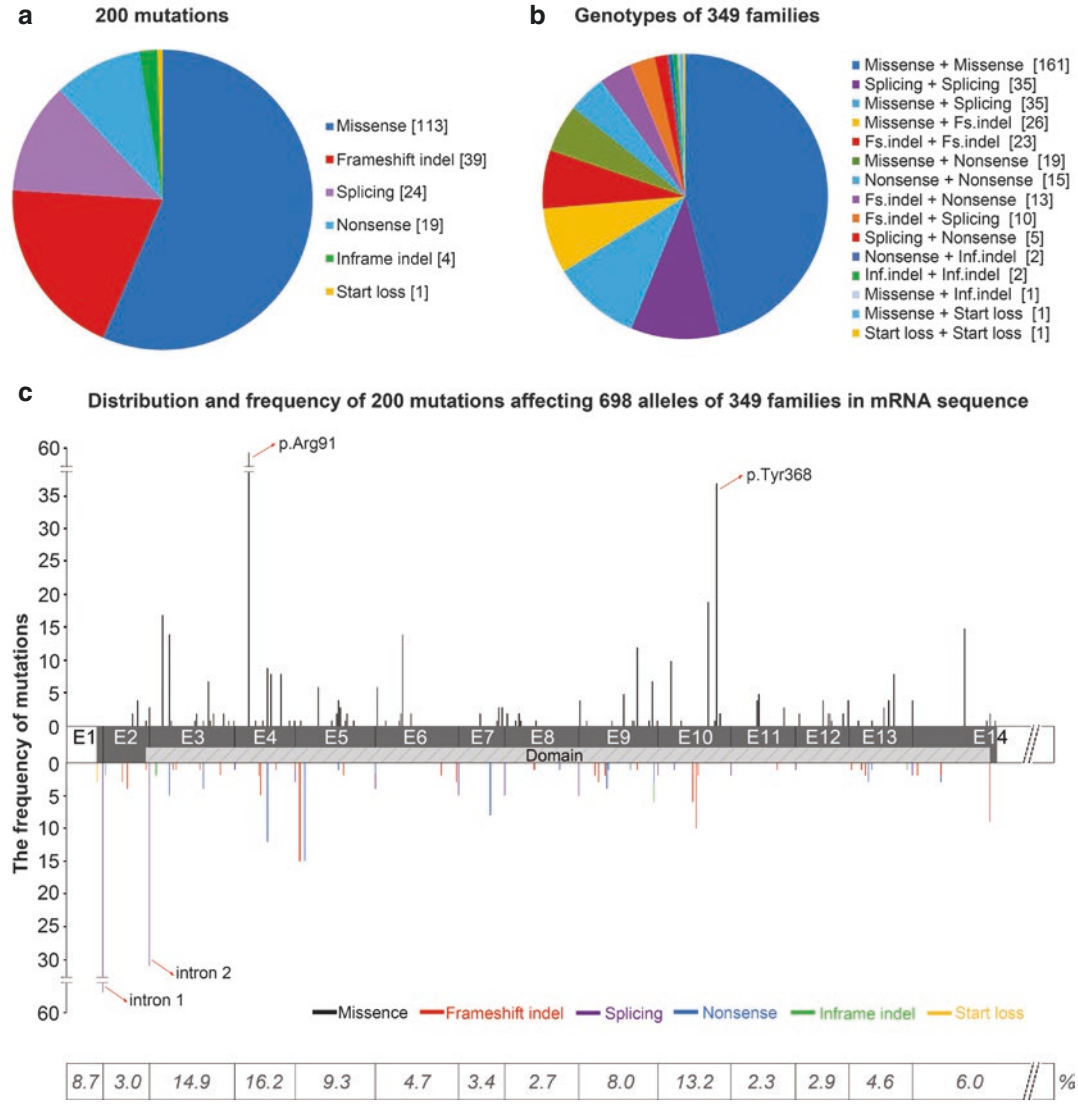

Fig. 14.1 Mutation spectrum and frequency in 349 families with biallelic *RPE65* mutations. (**a**) Two hundred mutations were identified in 349 families with biallelic *RPE65* mutations. The "frameshift indel" includes 20 frameshift deletion mutations and 19 frameshift insertion mutations. The "inframe indel" includes 3 inframe deletion mutations and 1 inframe insertion mutation. Splicing, splicing defect. (**b**) Genotypes of 349 families with biallelic *RPE65* mutations. Fs.indel, frameshift insertion or frameshift deletion mutations. Inf.indel, inframe insertion or inframe deletion mutations. (**c**) Distribution and frequency of 200 mutations affecting 698 alleles in 349 families shown in the mRNA sequence. Nucleotide numbering is based on the cDNA sequence of *RPE65* (Ref. NM_000329.2), where A of the ATG initiation codon is 1. The dark gray area in the middle represents the cDNA sequence. The light gray domain region extends from codon 30 to codon 530. The white areas before and after the cDNA sequence represent 5′ UTR and 3′ UTR, respectively. Partial sequences of 5′ UTR are omitted by double-dotted slashes. The position and frequency of the missense mutations are drawn above the structure of the mRNA, while other mutations are indicated below the structure of the mRNA by different colors. The frequency of p.Arg91 is 60, including 51 c.271C>T (p.Arg91Trp), eight c.272G>A (p.Arg91Gln), and one c.272G>C (p.Arg91Pro). The frequency of p.Tyr368 is 37, including 36 c.1102T>C (p.Tyr368His) and one c.1103A>G (p.Tyr368Cys). The frequency of intron 1 is 59, including two c.11+1G>T, 56 c.11+5G>A, and one c.12-2A>G. The frequency of intron 2 is 31, including two c.95-3C>G, one c.95-2A>C, 26 c.95-2A>T, and two c.95–1G>A. The frequencies of p.Arg91, p.Tyr368, intron 1, and intron 2 account for 26.8% of the total. The bottom numbers represent the percentage of allele frequencies per exon. Mutation hot spots in this gene are not apparent. E, exon

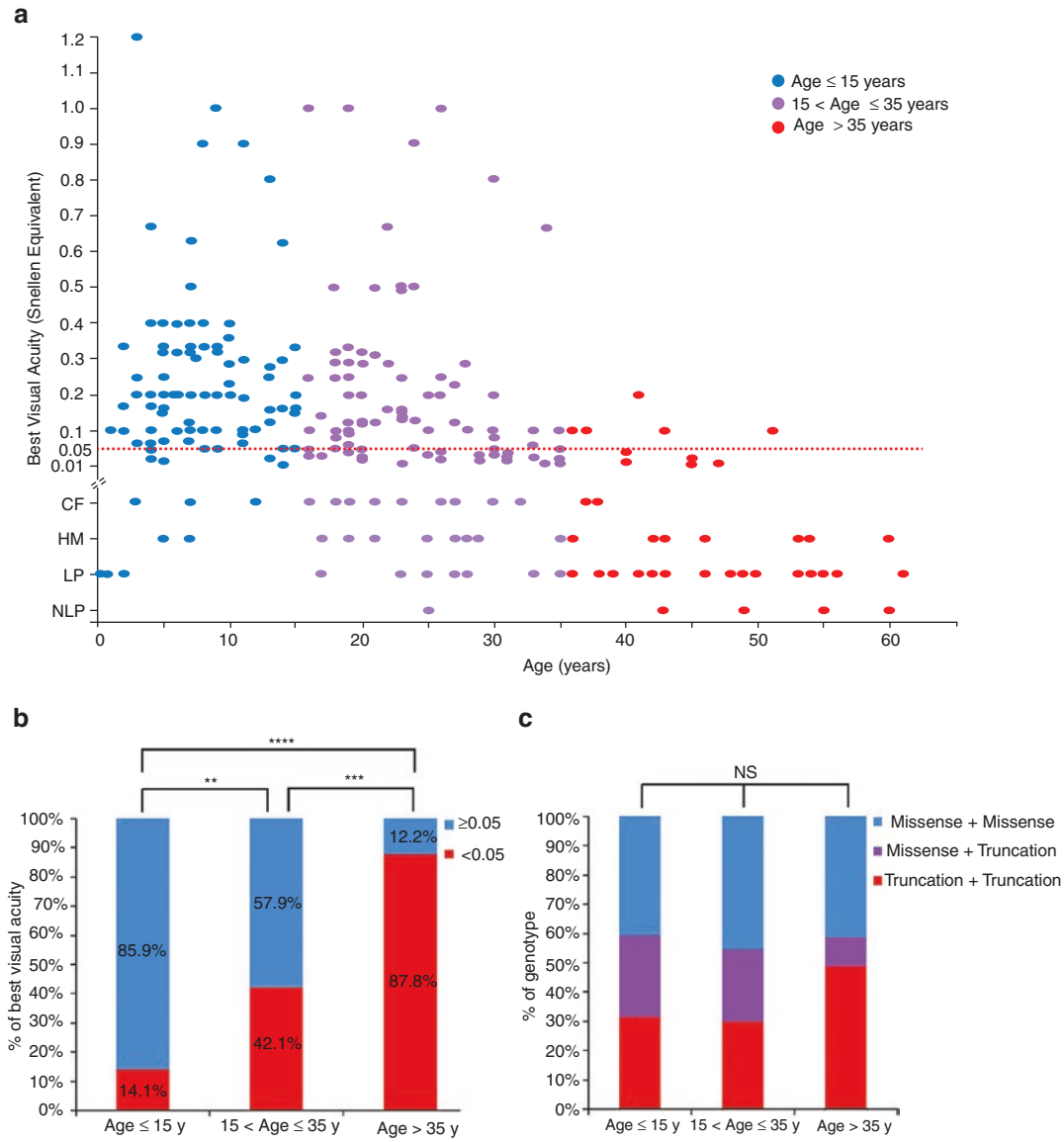

Fig. 14.2 Best visual acuity changing with age. (**a**) Best visual acuity for the better-seeing eye in patients with biallelic *RPE65* mutations, showing worsening acuity with aging. The red dashed line represents visual acuity of 0.05, which is the limit of legal blindness. CF, count finger; HM, hand motions; LP, light perception; NLP, no light perception. (**b**) Differences in best visual acuity in different age subgroups. $N = 99$ for the "Age ≤ 15 years" cohort, $N = 121$ for the "15 35 years" cohort. ** $P = 6.0E\text{-}6$, Chi-square test. *** $P = 3.9E\text{-}7$, Chi-square test. **** $P = 1.1E\text{-}16$, Chi-square test. y, years. (**c**) Genotypic differences among different age groups. $N = 99$ for the "Age≤15 years" cohort, $N = 121$ for the "15 35 years" cohort. NS, no significance; y, years

lower than 0.05. Therefore, these measurements could be classified into 3 subgroups according to age: younger than 15 years, 15 to 35 years, and older than 35 years. The proportion of patients with best visual acuity worse than 0.05 was significantly lower in the 15 to 35-year-old subgroup than in the older than 35 years subgroup, followed by that in the younger than 15 years subgroup, while the proportion with best visual acuity better than 0.05 showed the opposite trend

(Fig. 14.2b). There was no significant difference among the genotypes of these three subgroups (Fig. 14.2c), indicating that the age is a more reasonable explanation for the differences in vision.

14.3.4 Fundus Features of *RPE65* Mutated Patients

The available fundus photographs and corresponding ages were given for 67 patients. Reviewing features of these fundus photographs, they could be classified into 5 subgroups: no apparent pigment, white dots, atypical pigment, macular lesions mainly, and typical pigment (Fig. 14.3a). After analyzing the ages of patients with different fundus features, we detected age-dependent fundus changes. Patients with typical pigmentary changes in their fundus were mostly older than 35 years, while patients with no apparent pigment and white dots were mostly younger than 20 years. Once more, there was no significant difference among the genotypes of these three subgroups (Fig. 14.3b), indicating that the age of the patients, rather than the genotype, influences the fundus. Patients with typical pigmentary changes in their fundus were mostly older than 35 years, and patients whose findings were restricted to no apparent pigment and white dots were mostly younger than 20 years, suggesting that patients with typical pigmentation in their fundus prior to the age of 20 might not be due to *RPE65* mutations.

14.3.5 Genotype–Phenotype Correlations

The 349 homozygous or compound heterozygous families are composed of 230 families with LCA, 57 families with RP, 27 families with EOSRD, 6 families with FA, and 29 families with other diseases (Fig. 14.4a). Despite the different diagnoses, the ages of onset for all cases were very early (younger than 10 years old). The genotypes of these families could be classified into three subgroups based on the mutation types of both alleles: biallelic missense (missense + missense), biallelic truncation (truncation + truncation), and mixed missense + truncation. Truncation mutations include all other mutation types except missense mutations. There was no significant difference between the phenotypes of patients with truncation mutations causing nonsense-mediated decay and patients with truncation mutations escaping nonsense-mediated decay. Biallelic missense mutations were the most common genotype, followed by biallelic truncation mutations in *RPE65* (Fig. 14.4b). The distribution shown here was well outside of Hardy–Weinberg equilibrium, suggesting that a significant fraction of the cases might be consanguineous, or at least from isolated population groups. Though there was no significant difference among the genotypes of different VA subgroups or different fundus subgroups (Figs. 14.2c and 14.3b), comparison of the genotypes of different diagnosis subgroups (LCA, EOSRD, and RP patients) showed that LCA is significantly associated with biallelic truncation mutations and that RP is significantly associated with biallelic missense mutations (Fig. 14.4c). Thus, there is a direct correlation between more severe genotypes and more severe phenotypes.

Among the 200 biallelic mutations in *RPE65*, five missense mutations are with homozygous occurrences in the ExAC database. They are c.295G>A (p.Val99Ile), c.394G>A (p.Ala132Thr), c.746A>G (p.Tyr249Cys), c.963T>G (p.Asn321Lys), and c.1301C>T (p.Ala434Val). The c.963T>G (p.Asn321Lys) mutation was reported in five homozygous families and two compound heterozygous families in seven studies, with diagnoses ranging from RP to cone-rod dystrophy to LCA. The c.394G>A (p.Ala132Thr) mutation was reported in three homozygous families in three studies, with diagnoses ranging from RP to CORD to LCA. The remaining three mutations were reported in one compound heterozygous family, respectively. The c.295G>A (p.Val99Ile) mutation was reported in one high hyperopia family, c.746A>G (p.Tyr249Cys) in one EOSRD family, and c.1301C>T (p.Ala434Val) in one RP family.

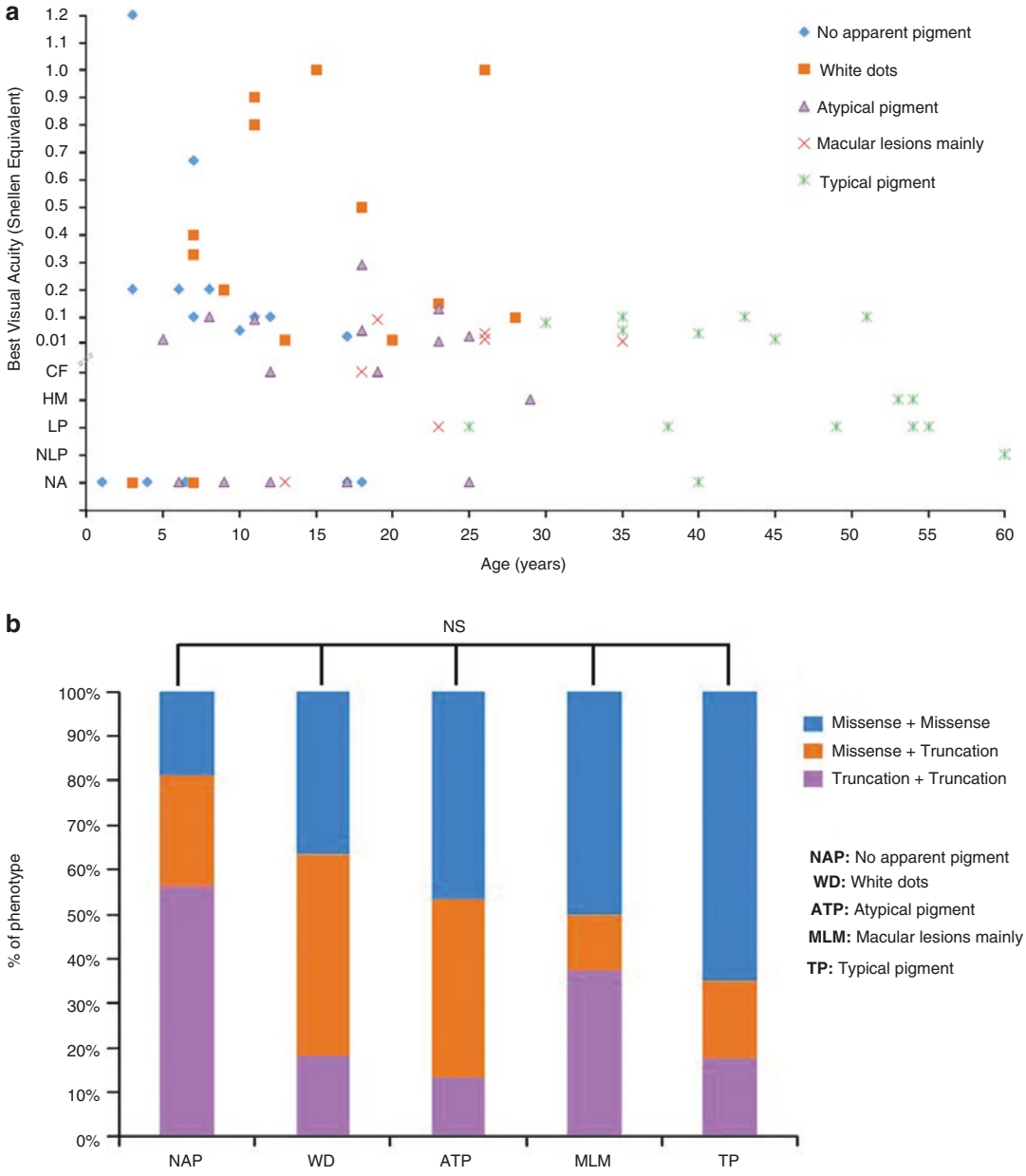

Fig. 14.3 Fundus features of *RPE65* mutated patients. (**a**) Age range and best visual acuity of patients with different fundi. CF, count finger; HM, hand motions; LP, light perception; NLP, no light perception. (**b**) Genotypic differences among different fundus subgroups. $N = 16$ for the "No apparent pigment" cohort, $N = 11$ for the "White dots" cohort, $N = 15$ for the "Atypical pigment" cohort, $N = 8$ for the "Macular lesions mainly" cohort, and $N = 17$ for the "Typical pigment" cohort. NS, no significance

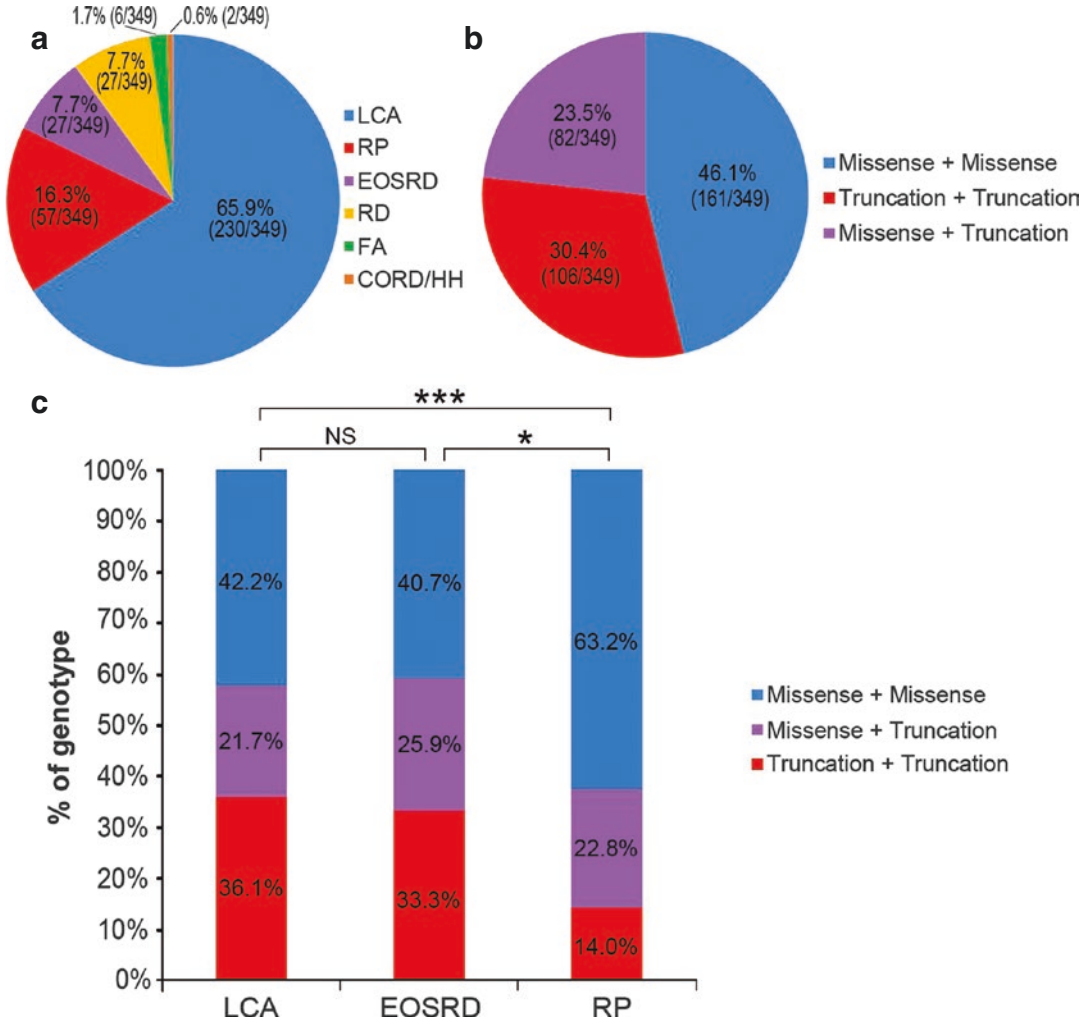

Fig. 14.4 The genotype–phenotype correlation in patients with biallelic *RPE65* mutations. (**a**) Phenotypic distribution of 349 families. LCA, Leber congenital amaurosis; RP, retinitis pigmentosa; EOSRD, early-onset severe retinal degeneration; RD, retinal dystrophy; FA, fundus albipunctatus; CORD, cone–rod dystrophy; HH, high hyperopia. (**b**) Genotypic distribution of 349 families. (**c**) Genotypic differences among different phenotypic cohorts. $N = 230$ for the LCA cohort, $N = 27$ for the EOSRD cohort, and $N = 57$ for the RP cohort. *** $P = 9.6E\text{-}4$, Mann-Whitney test; * $P = 3.0E\text{-}2$, Mann-Whitney test; NS, no significance, Mann-Whitney test. LCA, Leber congenital amaurosis; EOSRD, early-onset severe retinal degeneration; RP, retinitis pigmentosa

14.3.6 The Global Distribution of Families with Biallelic *RPE65* Mutations and Genotypic/Phenotypic Differences Among Different Ethnic Groups

When categorized by the sources of patients described in the literature or if unavailable, by the country of the corresponding author, the 349 families could be classified as originating in 29 countries worldwide (Fig. 14.5a). Of the 29 countries, the United States, China, the United Kingdom, India, and Italy were the most common countries, accounting for 61.0% of the 349 families together, although this finding might be influenced by the intensity of the research in those countries. The ethnic groups of these families can be classified into three subgroups based on countries and/or races: Caucasian, Asian, and African (Fig. 14.5b). Compared with Caucasian populations, the proportions of RP and biallelic missense were significantly higher in Asian ethnic groups (Fig. 14.5c, d). However, there was no significant difference between genotypes of the same disease subgroup (LCA or RP patients) in Caucasian and Asian

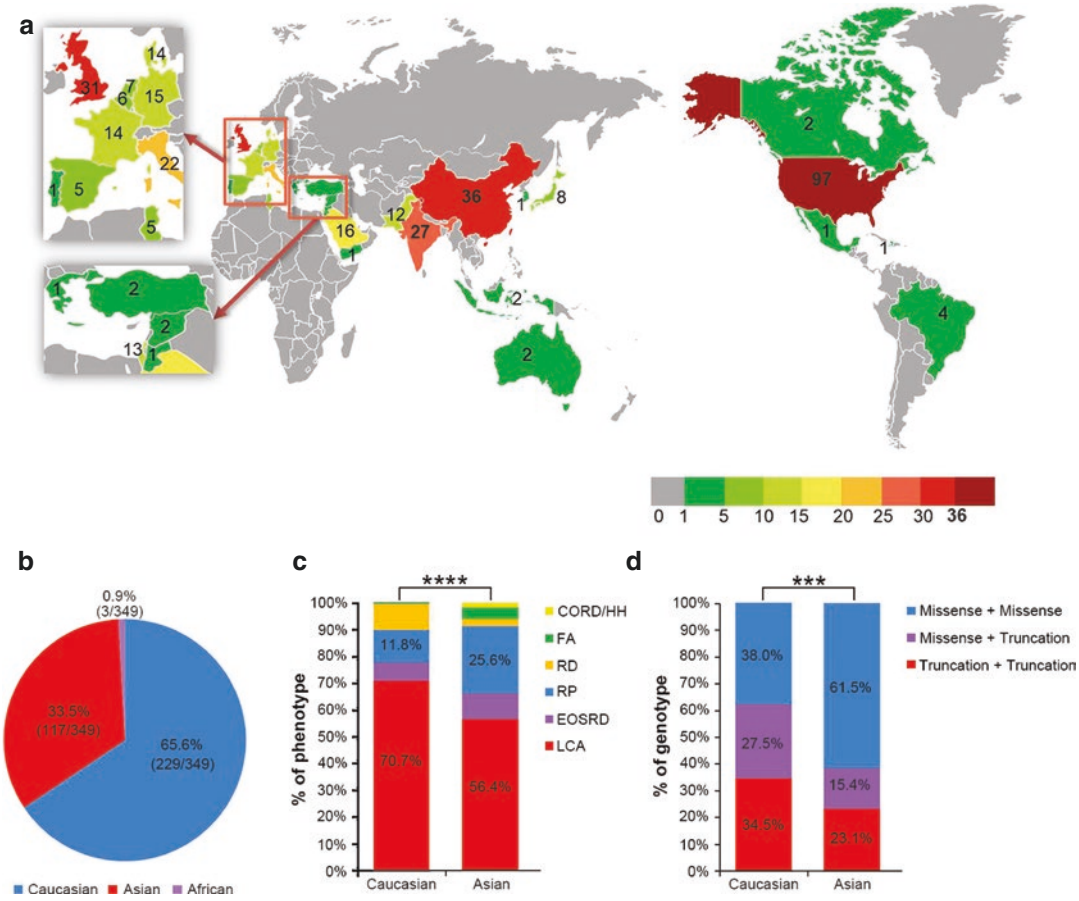

Fig. 14.5 The global distribution of 349 families with biallelic *RPE65* mutations and genotypic/phenotypic differences among different ethnic groups. (**a**) The global distribution of 349 families. The numbers represent the family frequency of the corresponding country. (**b**) The ethnic distribution of 349 families. (**c**) Phenotypic differences between Caucasian and Asian cohorts. $N = 229$ for the Caucasian cohort, $N = 117$ for the Asian cohort. **** P = 1.8E-5, Fisher's exact test. LCA, Leber congenital amaurosis; RP, retinitis pigmentosa; EOSRD, early-onset severe retinal degeneration; RD, retinal dystrophy; FA, fundus albipunctatus; CORD, cone–rod dystrophy. (**d**) Genotypic differences between Caucasian and Asian cohorts. $N = 229$ for the Caucasian cohort, $N = 117$ for the Asian cohort. *** P = 2.2E-4, Mann-Whitney test

patients (data not shown), indicating that the phenotypic differences between Caucasian and Asian patients relate primarily to the different classes of mutations in these groups.

14.4 Discussion

In this study, we summarized *RPE65* mutational profiles, clinical features of patients with *RPE65* mutations, and the genotype–phenotype correlation for IRDs.

For mutational profiles, we reviewed 201 mutations detected in 353 families with heterozygous or biallelic *RPE65* mutations. Among the four most common mutations, the mutation c.95-2A>T in intron 2 represents a founder mutation in a North African Jewish Population [63]. Unlike *CYP4V2* where several common variants were found in most patients [120], mutations in *RPE65* were evenly distributed on all 14 exons and mutation hot spots in this gene are not apparent, which is in accordance with a previous report [121]. Among the 200 mutations detected in families with biallelic *RPE65* mutations, most of them have low allele frequencies in existing databases (ExAC and 1000 Genomes) and were predicted to be damaging by various pathogenicity prediction tools. However, 13 changes identified as mutations in the literature were predicted to be benign by both SIFT and PolyPhen-2. Biallelic mutations in *RPE65* that include one predicted to be benign are likely to be associated with milder phenotypes. Pairs of mutant *RPE65* alleles of which both were predicted to be benign, such as c.963T>G (p.Asn321Lys), have been reported to be disease-causing in multiple papers. In addition, our own data have shown that biallelic variants in *RPE65* are very rare [5]. Thus, biallelic variants in *RPE65* in which both variants are predicted to be benign by SIFT and PolyPhen-2 should not be excluded rashly during clinical genetic testing. The population frequency and the prediction results of more tools that predict pathogenicity should be considered. Though the five missense variants with homozygous occurrences in the ExAC database have relatively high population allele frequencies and are predicted to be benign by multiple tools, they were reported to be disease-causing in multiple papers, such as c.394G>A (p.Ala132Thr). Patients should be informed about the uncertain pathogenicity of these variations during clinical genetic testing. In future studies, if patients with these variants are encountered, peripheral fundus examination, electroretinogram, and cosegregation analysis should be conducted, which are helpful for clarifying their pathogenicity.

Clinically, patients with biallelic *RPE65* mutations have the following clinical characteristics: the onset age was early (<10 years old); best visual acuity decreases with age; white dots or no apparent pigment fundus changes are present in patients younger than 20 years, while the typical pigmentary fundus changes are found mostly in patients older than 35 years. The clear trend of lower VA with increasing age is in accordance with previous reports [41, 48, 118]. The best visual acuity of patients with biallelic *RPE65* mutations is relatively good before 15 years old, indicating that these patients are unlikely to have a desire to undergo gene therapy. In contrast, the great majority of patients aged older than 35 years old are legally blind, and gene therapy is likely to be too late to improve their visual acuity. Patients aged between 15 and 35 years are most likely to receive gene therapy because of their decreasing visual acuity and their relatively curable photoreceptor function. In addition to best visual acuity, the fundus features of patients with biallelic *RPE65* mutations also change with age, which is valuable for clinical diagnosis. In addition, our previous research indicated that one-third of patients with *RPE65* mutations had white dots fundus [5]. Although only two FA cases were reported in previous literature, with the exception of our previous research, we have reviewed more patients with *RPE65* mutations with white dots fundus. This observation indicates that there may be many more patients with *RPE65* mutations with white dots fundus, which is one of the most important clinical features of patients with *RPE65* mutations. Numerous studies specifically searched for *RPE65* mutations in the LCA cohort, and fewer searched for other diseases, such as RP, especially in Caucasian popu-

lations. This finding suggests that there may be more IRDs patients with *RPE65* mutations that could be treated with gene therapy. In the case of limited genetic testing conditions, the *RPE65* gene can be specifically sequenced in patients with these clinical characteristics. Conversely, for patients whose clinical manifestations are contradictory to the above characteristics, such as late-onset age (>20 years old) and the presence of typical pigmentation fundus of the eyes before the age of 20 years, their phenotypes are less likely to be attributable to *RPE65* biallelic mutations.

Based on comparative genotypic analysis of patients with different phenotypes, we identified an association between biallelic missense mutations and RP, while biallelic truncating mutations tended to be associated with LCA. We speculate that the milder phenotype of RP might be due to some residual catalytic activity of *RPE65* isomerase in patients with biallelic missense mutations. The correlation between phenotypic severity and mutation type is helpful for gene testing. This discovery could be applied to both Caucasian and Asian populations because the phenotypic differences between Caucasian and Asian individuals tend to be due to their different *RPE65* genotypes rather than susceptibilities resulting from other genetic loci.

In summary, we reviewed all published *RPE65* mutations identified in patients with IRDs. Our results revealed the phenotypic characteristics of patients with *RPE65* mutations and their genotype–phenotype correlations, which will be helpful for clinical diagnosis and gene testing. Furthermore, the regularity of the decline in best visual acuity with age should be valuable for selecting patients for gene therapy and predicting its efficacy.

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Genetic Variants and Impact in PDE6B Rod-Cone Dystrophy

15

Christopher J. G. Watson, Benjamin M. Nash, To Ha Loi, John R. Grigg, and Robyn V. Jamieson

Abstract

Phosphodiesterase 6B (PDE6B) is one of the most commonly mutated genes to cause autosomal recessive retinitis pigmentosa (RP), also known as rod-cone dystrophy. The PDE6B protein plays a crucial role in the phototransduction cascade. With the emerging possibility of genetic therapy for autosomal recessive *PDE6B*-related retinitis pigmentosa, knowledge regarding the pathogenicity and functional significance of identified *PDE6B* variants is crucial for genetic information for families and access to clinical trials. We collected all *PDE6B* variants reported in 207 autosomal recessive RP patients in publica-

C. J. G. Watson
Eye Genetics Research Unit, The Children's Hospital at Westmead, Save Sight Institute, Children's Medical Research Institute, University of Sydney, Sydney, NSW, Australia

Disciplines of Genomic Medicine, and Child and Adolescent Health, Faculty of Medicine and Health, University of Sydney, Sydney, NSW, Australia

B. M. Nash
Eye Genetics Research Unit, The Children's Hospital at Westmead, Save Sight Institute, Children's Medical Research Institute, University of Sydney, Sydney, NSW, Australia

Disciplines of Genomic Medicine, and Child and Adolescent Health, Faculty of Medicine and Health, University of Sydney, Sydney, NSW, Australia

Sydney Genome Diagnostics, Western Sydney Genetics Program, The Children's Hospital at Westmead, Sydney, NSW, Australia
e-mail: Benjamin.nash@health.nsw.gov.au

T. H. Loi
Eye Genetics Research Unit, The Children's Hospital at Westmead, Save Sight Institute, Children's Medical Research Institute, University of Sydney, Sydney, NSW, Australia
e-mail: tloi@cmri.org.au

J. R. Grigg
Eye Genetics Research Unit, The Children's Hospital at Westmead, Save Sight Institute, Children's Medical Research Institute, University of Sydney, Sydney, NSW, Australia

Discipline of Ophthalmology, Faculty of Medicine and Health, University of Sydney, Sydney, NSW, Australia
e-mail: john.grigg@sydney.edu.au

R. V. Jamieson (✉)
Eye Genetics Research Unit, The Children's Hospital at Westmead, Save Sight Institute, Children's Medical Research Institute, University of Sydney, Sydney, NSW, Australia

Disciplines of Genomic Medicine, and Child and Adolescent Health, Faculty of Medicine and Health, University of Sydney, Sydney, NSW, Australia

Western Sydney Genetics Program, Department of Clinical Genetics, The Children's Hospital at Westmead, Sydney, NSW, Australia
e-mail: rjamieson@cmri.org.au

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tions before June 2019. The 101 unique variants obtained were classified according to the American College of Medical Genetics and Genomics guidelines. Our study provides information on the variant type, location, and predicted pathogenicity of the variants. It also highlights the need for ongoing review, as well as additional data from functional assays, to better understand the clinical significance of *PDE6B* variants.

Keywords

PDE6B · Retinitis pigmentosa · Retinal degeneration · Rod-cone dystrophy Pathogenic variants

15.1 Introduction

Retinitis pigmentosa (RP) covers a group of diseases causing progressive visual loss as a result of rod photoreceptor cell death. RP is the commonest form of retinal degeneration, with a prevalence of approximately 1 in 4000 [1, 2], with a significant burden of disease. In a Scandinavian prevalence study, RP was the equal first cause of blindness in citizens aged 20–64 years [3], while in Japanese rehabilitation centers, RP was the leading cause of visual handicap or blindness, affecting a quarter of visually affected patients [4]. The impact of inherited retinal dystrophies is highlighted by a UK study, which showed retinal dystrophies were the leading cause of blindness in working age individuals, even exceeding those due to diabetic retinopathy [5].

The inheritance pattern of RP can be autosomal dominant (approximately 15–25% of cases), autosomal recessive (5–20%), or X-linked (5–15%), and is unknown in approximately 40–50%, which are simplex cases [6–8]. RP is a highly heterogeneous disease. The clinical phenotype is variable, even in patients with an identical mutation of a disease gene [9, 10]. All forms lead to vision impairment, but there is extreme variability in rates of degeneration between families [11, 12]. The diagnostic criteria delineated by the 1982 International Symposium of Ophthalmology are [1] bilateral involvement, [2] concentric depression of the visual field, [3] severe scotopic involvement on electroretinogram (ERG), resulting from alteration of rods, or even no ERG response, and [4] progressive loss of photoreceptor function [13].

Genes associated with mutations in RP include those involved in the phototransduction cascade. Rod phosphodiesterase (PDE) is made up of a catalytic heterodimer of PDE6A and PDE6B, as well as two identical inhibitory gamma subunits [14]. This protein plays a crucial role in the phototransduction cascade, by hydrolyzing the second messenger cGMP as a response to light, resulting in photoreceptor channel closure [14]. Defects resulting from *PDE6B* mutations lead to high concentrations of cGMP and cell death [15, 16]. Mutations in the gene encoding the beta-subunit of rod PDE, *PDE6B* (MIM# 180072), account for 4–5% of autosomal-recessive cases [17–19].

The *PDE6B* gene is composed of a 3414 base pair long mRNA sequence encoding the beta-subunit of rod phosphodiesterase, an 854 amino acid long protein [20–22]. Its molecular mass is 98.4 kDa. The gene has 22 exons and is located on the short arm of chromosome 4 [23]. The PDE6B protein has two high-affinity non-catalytic binding (GAF) domains and one catalytic PDEase domain. Clinical information suggests *PDE6B* RP patients have features of typical RP which may include early nyctalopia and first presentation during childhood or early adulthood, but there has been little detailed information about disease progression [18, 21, 24–37]. In a retrospective study of clinical features, Khateb et al. analyzed *PDE6B* RP progression in 35 patients from 26 families. Fifteen novel genetic variants in *PDE6B* were also reported in this study [36]. The mean age at diagnosis was 21.1 years (range, 3–45 years), and nyctalopia was the most prevalent presenting symptom which was reported in 13 out of 35 patients (37%). The mean best corrected visual acuity (BCVA) at the first examination was 0.4 logMAR (Snellen equivalent: 6/15 [Metric]). With follow-up visual acuity data for 1 to over 15 years on 24 patients, the annual estimated mean rate of decline of BCVA was 2%,

compared with 1%, 2%, and 8.6% for other rod-cone dystrophy cohorts [12, 38, 39]. Goldmann kinetic visual field measurements exhibited sizable degrees of decrease over time in *PDE6B* mutation patients, and quantifiable hyperautofluorescent ring changes were also noted in these patients [36, 40].

The aim underpinning gene therapy in autosomal recessive retinal dystrophies, such as those due to variants in *PDE6B*, is to replace absent or inadequate functional protein products. There are two common murine models of autosomal recessive *PDE6B* RP that have been extremely useful in progress toward therapy for this condition. In the naturally occurring *rd1* mouse model, there is severe early-onset photoreceptor degeneration due to a nonsense point mutation that creates a stop codon in exon 7 of the mouse *Pde6b* gene p.(Tyr347Ter). Chang et al. first reported the *rd10* mouse in 2000 [41, 42], another naturally occurring murine model of *Pde6b* RP. The pathogenic missense mutation is p.(Arg560Cys), in exon 13 of mouse *Pde6b*. The *rd10* mouse has a later onset of disease and detectable levels of PDE6B protein, and has proven more useful in modelling gene therapy, as its disease course is closer to that in human RP. Several gene therapy experiments have been conducted in these mouse models [43, 44], including gene therapy through a subretinal injection of an AAV2/5 vector, which was shown to delay rod degeneration and maintain ERG response for at least 3 weeks after treatment [45].

In naturally occurring *PDE6B*-deficient rod-cone dysplasia type 1 (*rcd1*) dogs, a large animal model of RP with a p.(Trp807Ter) mutation, subretinal AAV-mediated gene therapy restored dim light vision, preserved retinal cell structure, and increased electroretinography rod signals for at least 40 months [46, 47]. Dogs were treated with either AAV2/5RK-cpde6β or AAV2/8RK-cpde6β. In this same model with the same treatment, gene therapy arrests the degenerative process even if it is given after the onset of photoreceptor degeneration [47]. AAV2/5 and AAV2/8 vectors can efficiently induce gene transduction if injected sub-retinally in dogs, and there is preliminary evidence that AAV2 could confer gene transduction if delivered intravitreally [48]. This is a promising finding in terms of future clinical trials.

These animal model studies were the prelude to establishment of a replacement gene therapy clinical trial for patients with autosomal recessive rod-cone dystrophy due to *PDE6B* molecular defects (https://clinicaltrials.gov/ct2/show/results/NCT03328130). To gain any benefit from gene replacement therapy for *PDE6B*-related retinal dystrophy, it would be expected that the molecular defects would be biallelic compound heterozygous or homozygous variants causing decreased function of the PDE6B protein. In view of this requirement, it is useful to consider the current classification of reported *PDE6B* variants in the literature, and potential prospects on how to assess the functional significance of variants. Facilitation of better interpretation of the pathogenicity of *PDE6B* variants in relation to autosomal recessive RP, will be important in consideration of *PDE6B*-related RP clinical trial eligibility.

15.2 Materials and Methods

We obtained all studies published prior to June 2019 that reported *PDE6B* variants in autosomal recessive RP patients. Demographic information, age of onset, and disease phenotype were collected if reported. Clear duplicates were removed.

We collected Polymorphism Phenotyping v2, SIFT, and PMut scores for all missense variants. Polymorphism Phenotyping v2 (PolyPhen-2) scores were obtained from genetics.bwh.harvard.edu/pph2/ [49]. SIFT scores were obtained from http://siftdna.org [50], using the GRCh37/hg19 assembly. Lastly, PMut scores were obtained from mmb.pcb.ub.es/Pmut [51]. Computational scores for splice site variants were obtained using the programs NNSPLICE, Max-EntScan, and Human Splice Finder, accessed via Alamut Visual 2.8 (Interactive Biosoftware, Rouen, France).

The reported *PDE6B* variants were each allocated a predicted pathogenicity classification according to the American College of Medical Genetics and Genomics (ACMG) guidelines [52]. The classifications were: pathogenic, likely

pathogenic, variant of uncertain significance, likely benign, and benign.

15.3 *PDE6B* Variants

15.3.1 Spectrum of *PDE6B* Variants

We collected data on 207 autosomal recessive RP patients reported in 43 papers [15, 17–19, 21, 24–37, 53–76], with a total of 367 reported alleles containing *PDE6B* variants. In this group, 111 patients had homozygous *PDE6B* variants, 49 patients had compound heterozygous variants, and 47 patients carried one variant. The 367 alleles consisted of 101 unique *PDE6B* variants, with missense variants as the most common type (189 alleles of 45 unique variants), followed by protein-truncating variants (104 alleles of 37 unique variants), and then splice site variants (72 alleles of 17 unique variants) (Fig. 15.1a, b and Table 15.1). Of the protein-truncating variants, 43 alleles had nonsense mutations and 61 caused a frameshift. In addition, two unique complex *PDE6B* alleles were described in two different patients from the same study: c.[1401+4_1401+16delins14; 2326G>A] and c.[1401+4C>T; 2326G>A], [25] each encoding a splice site mutation and the missense mutation p.(Asp776Asn). The two splice site variants have not been reported in any other RP patient, and while the c.(2326G>A) variant was present in both these patients, it has not been reported elsewhere [25].

Table 15.1 *PDE6B* variant types reported in autosomal recessive RP patients

Variant type	Unique variants	Total number of alleles
Missense	45	189
Protein-truncating	37	104
Splice site	17	72
Complex alleles	2	2

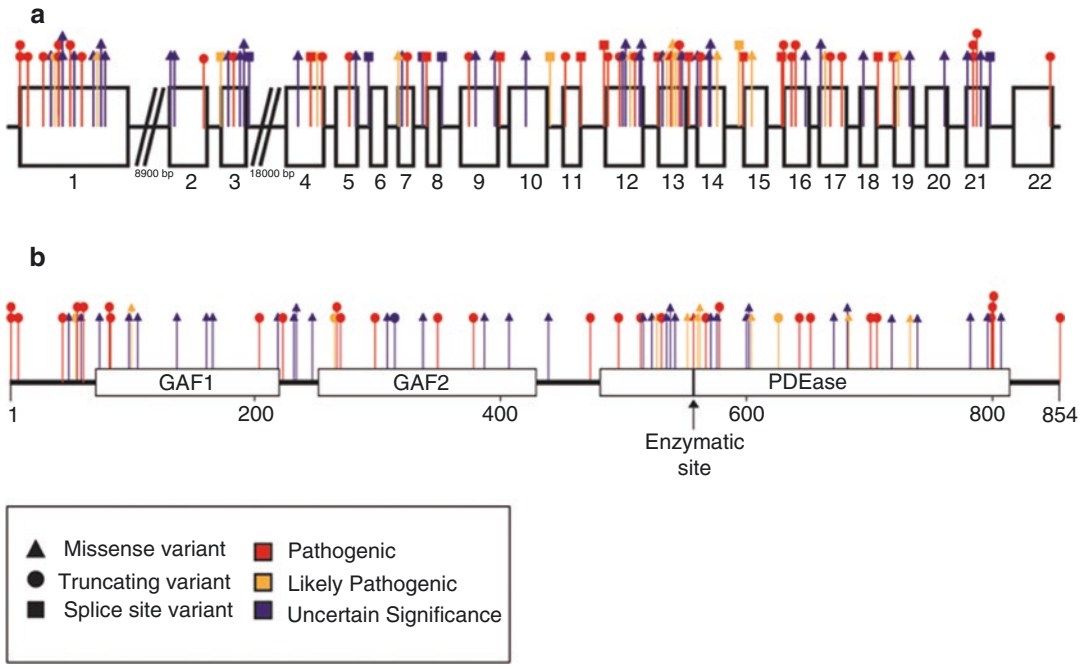

Fig. 15.1 Schematic representation of the locations of all reported *PDE6B* variants. (**a**) Exon–intron schematic. (**b**) Functional domain schematic. Missense variants are depicted as triangles, protein-truncating variants as circles, and splice site variants as squares. Pathogenic variants are red, Likely Pathogenic variants are yellow, and Variants of Uncertain Significance are blue. Accession reference NM_000283

The variant that affected the most autosomal recessive RP patients was p.(His557Tyr), which was reported in 21 alleles of 16 patients. This missense mutation was mainly found in Korean patients, but was also present in patients from Japan, China, and the USA. The most frequently detected variant in RP patients was p.(Arg552Gln), another missense mutation with homozygous expression in 24 alleles of 13 patients. Most of these patients were from Pakistan. Both frequent variants are found on exon 13, which had the most reported variants (61 alleles of 7 unique variants) out of all the exons. Notably, exon 13 encodes the catalytic site of the PDE6B protein (https://pfam.xfam.org). Exon 1 contained the second-most reported variants, and the highest number of unique variants (51 alleles of 18 unique variants). Exon 8 was the only exon to have no reported variants. Regarding splice site intronic variants, intron 15 had the most reported variants (18 alleles of 2 unique variants), and intron 10 had the highest number of unique intronic variants (4 alleles of 3 unique variants). Introns 2, 3, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 18, and 21 all also contained reported variants.

15.3.2 Pathogenicity Assessment of *PDE6B* Variants

The PDE6B variants were classified according to the ACMG guidelines. This led to the classification of 45 variants as pathogenic, 16 as likely pathogenic, and 38 as variants of uncertain significance, in addition to the two complex alleles.

The most clear-cut variants for pathogenicity classification were those that were predicted to lead to protein truncation. In this group, there were 104 alleles of 37 unique variants reported. Of the 37 unique variants, 19 were nonsense mutations and 18 were frameshift mutations. Thirty-four of the truncating variants were classified as Pathogenic or Likely Pathogenic. There were no reported patients with a variant orthologous to the *rd1* mouse p.(Tyr347Ter) mutation. Interestingly, de Castro-Miro et al. identified an RP patient with a heterozygous variant, p.(Tyr314CysfsTer50), in the heterozygous state [60], which was previously linked to autosomal dominant CSNB [77]. De Castro-Miro et al. considered this allele to be unlikely to be causing autosomal dominant disease in their family, because it was also present in heterozygous form in three other family members who were all clinically unaffected [60]. Hence, it is possible that their patient actually had autosomal recessive disease with a variant in their other *PDE6B* allele that was not able to be detected by the sequencing methodology of their study. De Castro-Miro et al. also found this patient to be heterozygous for a variant in *USH2A*, another known cause of autosomal recessive RP [60]. Hence, it is also possible this patient's disease could be due to presence of this allele and another *USH2A* allele that was not able to be detected by the sequencing methodology of their study. In addition, the suggested link of the p.(Tyr314CysfsTer50) variant with autosomal dominant CSNB may be rather tenuous, since there is only clinical ophthalmic data on the proband, and no variant data available on the reportedly affected deceased parent and grandparent [77]. These considerations highlight the need for careful review of segregation and phenotypic data, to help in review of variant classification and consideration of contribution to disease phenotype.

Splice site variant pathogenicity classification was also clear-cut when the variants affected the canonical splice site regions. There were 72 reported splice site alleles, with 17 unique variants. Of these, 10 unique variants affected the canonical dinucleotides of the splice acceptor (AG) or splice donor (GT) site, with 9 being classified as Pathogenic according to the ACMG guidelines, and one as Likely Pathogenic.

The missense alleles are the group where variants are most frequently classified as of uncertain significance, due to insufficient criteria according to the ACMG guidelines to classify them otherwise. A total of 189 alleles with *PDE6B* missense variants were reported in the autosomal recessive RP patients. Of the 45

Table 15.2 Likely pathogenic and pathogenic *PDE6B* missense variants

cDNA change	Protein change	Homozygous	Heterozygous	Domain
c.(299G>A)	p.(Arg100His)	–	2	GAF1
c.(1010A>G)	p.(His337Arg)	4	4	GAF2
c.(1580T>C)	p.(Leu527Pro)	–	3	PDEase
c.(1655G>A)	p.(Arg552Gln)	11	–	PDEase
c.(1669C>T)	p.(His557Tyr)	5	11	PDEase, enzymatic site
c.(1678C>T)	p.(Arg560Cys)	2	2	PDEase
c.(1685G>A)	p.(Gly562Asp)	3	–	PDEase
c.(1811C>T)	p.(Thr604Ile)	–	10	PDEase
c.(2047G>A)	p.(Val683Met)	–	2	PDEase
c.(2197G>C)	p.(Ala733Pro)	9		PDEase

unique missense variants, one was classified as Pathogenic, 9 as Likely Pathogenic, and 35 as Variants of Uncertain Significance (Table 15.2). In order to apply the ACMG guidelines, we obtained PolyPhen-2, SIFT, and PMut scores, and set the thresholds for pathogenicity at scores of >0.85, <0.05, and >0.50, respectively. These in silico predictions were taken as a piece of evidence only if all three computational scores were congruent. We investigated whether the mutations were located in a mutational hot spot or a well-established functional domain. The Pfam prediction tool (https://pfam.xfam.org) identifies two high-affinity non-catalytic binding (GAF) domains and one catalytic PDEase domain (Fig. 15.1b) [78]. While GAF domains are present in cGMP-specific phosphodiesterases and phytochromes, such regions can be difficult to strictly define as a "mutational hot spot" as per the ACMG guidelines, since there is an implied requirement that all missense variants in such domains have been shown to be pathogenic [52]. This criterion is difficult to use in the clinical setting, as there will often be newly identified variants in a region that may not have reached full classification criteria due to eg lack of available parental segregation data or other affected family members to test. However, we concluded that the variant encoding p.(His557Tyr) was located in a critical location, as the enzymatic site of PDE6B is found at p.(557) (https://pfam.xfam.org). Four patients had the missense variant p.(Arg560Cys), which is orthologous to the mutation in the *rd10* mouse.

15.3.3 Genotype–Phenotype Correlation

We collected data on age of onset, disease phenotype, gender, and ethnicity for any autosomal recessive RP patient with a reported *PDE6B* variant. However, there was little detailed phenotypic data reported for the majority of patients, and we could not investigate a possible genotype–phenotype correlation. It was therefore not possible to evaluate whether the variant type or location had an impact on disease severity. While no statistical analysis was possible, we did note the high variability in phenotype. For example, age of onset ranged from infancy to adulthood [18, 21, 24–37]. This variability highlights the importance of detailed phenotype reporting, so that any potential genotype–phenotype correlation may be uncovered. The retrospective review undertaken by Khateb et al. showed useful detailed ophthalmic phenotype data over a period of more than 15 years in some cases [36]. Such information is useful in informing the most appropriate timing for therapeutic intervention, outcome measures, and required duration of follow-up for future *PDE6B*-related clinical trials.

15.3.4 Future Directions

It should be noted that there may be variation in interpretation of ACMG classification criteria and these may vary across groups and may be subject to change over time. Many of the *PDE6B* mis-

sense variants, and several others, were classified as Variants of Uncertain Significance according to the ACMG guidelines. In these cases, it would be useful to evaluate pathogenicity with functional experiments. Unfortunately, there is little reported functional experimental evidence for most of the *PDE6B* variants. *PDE6B* mRNA expression is low in all tissue types except the retina, meaning that easily collected patient cells may not be useful in functional studies [79]. One study collated in this work used a splice assay to determine the pathogenicity of a variant, and this was a mini-gene-based splice assay of a novel intronic variant [37].

There are several ways in which the molecular effects of the variants could be investigated. Further, mini-gene-based splice assays could be used for investigation of possible splice variants. Alternatively, patient-derived human induced pluripotent stem cells could be differentiated to retinal organoids [80], and used for RNA extraction and variant splice-form expression studies. In mouse retinal cells, it is possible to assess PDE activity by measurement of cGMP levels [81]. A possible avenue may be to use *PDE6B* variant patient-derived ocular organoids to assess cGMP levels and impact on morphological and expression characteristics of the organoids.

15.4 Conclusion

We have collated all *PDE6B* variants from published studies of patients with autosomal RP and assessed their predicted pathogenicity according to the ACMG guidelines. While this contribution is based on known variants, we anticipate that more variants will be discovered. Along with our data, genetic and phenotypic information published in the future may cast light on any genotype–phenotype correlation, as well as providing additional evidence for the classification of variants. There is a need for functional experiments that would become another source of evidence. Taken together, these data would improve our understanding of the different *PDE6B* variants. In addition, these approaches will be critical in improving diagnostic accuracy for variants in conditions where eligibility for therapeutic possibilities requires certainty around the genetic diagnosis.

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The Retinitis Pigmentosa Genes

16

Xue Chen and Chen Zhao

Abstract

Retinitis pigmentosa (RP), the most common form of inherited retinal dystrophies (IRDs), is a monogenic disease with remarkable genetic heterogeneities. All three types of Mendelian inheritance patterns have been found associated with RP, including autosomal dominant, recessive, and X-linked modes. By far, 87 genes and 7 loci have been linked to RP. These genes show variable expression patterns and are involved in multiple biological pathways, such as phototransduction cascade, visual cycle, ciliary structure and transport, and so on. In this chapter, we will talk about genes involved in RP etiology. Currently, no generally applicable treatment has been developed for RP, therefore better insights into the RP etiology will help with better management of RP patients.

Keywords

Retinitis pigmentosa · Genes
Phototransduction cascade · Visual cycle
Ciliary structure and transport

16.1 Introduction

Retinitis pigmentosa (RP) is the most common form of inherited retinal dystrophies (IRDs), presenting a prevalence ranging from 1/750 to 1/9000 among different populations [1]. RP is characterized by photoreceptor degeneration and pigment migration. Rod photoreceptors and/or retinal pigment epithelium (RPE) cells are affected in the initial stage of the disease, and cone photoreceptors are involved at a later stage. Typical symptoms of RP patients include night blindness, visual field constriction, and eventual loss of central vision [2]. Fundus abnormalities are bone spicule pigmentation predominantly in the periphery and/or mid-periphery retina, attenuated retinal vessels, and a waxy pallor of the optic disc. Electroretinogram (ERG) can help with the diagnosis and reveal the photoreceptor dysfunction. Noteworthy, patients with some systemic diseases, like Usher syndrome and Bardet-Biedl syndrome, may also have RP presentations. In this chapter, we only talk about the non-syndromic form of RP.

X. Chen
Department of Ophthalmology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China

C. Zhao (✉)
Department of Ophthalmology and Vision Science, Eye & ENT Hospital, Shanghai Medical College, Fudan University, Shanghai, China

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16.2 Genes Involved in RP

RP can be inherited via all three types of Mendelian inheritance, including autosomal dominant, recessive, and X-linked modes. Digenic and incomplete dominant forms have also been reported [114, 115]. RP shows remarkable genetic heterogeneity. To date, 87 genes and 7 loci have been linked to RP (see the Retinal Information Network [RetNet] at https://sph.uth.edu/RetNet/) (Table 16.1). However, these genes only account for the etiology of approximately 60% of all RP patients, indicating that lots of novel RP causing genes are still to be identified. Each of the 87 genes encodes a protein that plays crucial roles in maintaining retinal homeostasis, such as sustaining phototransduction cascade and visual cycle. Mutations in those genes would impair the encoded protein and their relevant pathway, thus further disrupting regular retinal function. In this chapter, we will talk about the 87 genes and principle pathways that are affected in RP (Table 16.2). Specifically, we will focus on the phototransduction cascade, the visual cycle, and ciliary structure and transport.

16.3 The Phototransduction Cascade

The phototransduction pathway is a cascade of reactions triggered by excitation of the opsin molecule by a photon, thus generating an electrical signal that is transmitted through the optic nerve to the visual cortex. In rod photoreceptors, the chromophore 11-*cis*-retinal converts to the all-*trans*-retinal isomer when capturing a photon, which then changes the structure of rhodopsin (encoded by the *RHO* gene) into the photoactive metarhodopsin II [116]. The G protein transduction (encoded by the *GNAT1* gene), activated by the metarhodopsin II, further activates the cyclic guanosine monophosphate (cGMP) phosphodiesterase (with subunits encoded by the *PDE6A*, *PDE6B*, and *PDE6G* genes), thus turning cGMP into 5′-GMP and shutting down cGMP-gated channels (with subunits encoded by the *CNGA1* and *CNGB1* genes) in the plasma membrane of photoreceptors [117]. Closure of the cGMP-gated channels decreases the intracellular calcium concentration and subsequently hyperpolarizes the plasma membrane, which would cause decreased glutamate release at the photoreceptor's synapse.

Photoreceptors will go back to the pre-photoactivation status after phototransduction via several ways. Firstly, activated rhodopsin kinase phosphorylates metarhodopsin II, which then binds arrestin (encoded by the *SAG* gene) and deactivate the phototransduction [118, 119]. Secondly, all-*trans*-retinal dissociates from the visual pigment and converts to 11-*cis*-retinal though the visual cycle. Furthermore, GTPase-accelerating proteins, such as RGS9, inhibit

Table 16.1 Summary of RP genes

Inheritance mode	No. of genes and loci	Mapped loci (not identified)	Mapped and identified genes
Autosomal dominant	30	RP63	*ADIPOR1, ARL3, BEST1, CA4, CRX, FSCN2, GUCA1B, HK1, IMPDH1, KLHL7, NR2E3, NRL, PRPF3, PRPF4, PRPF6, PRPF8, PRPF31, PRPH2, RDH12, RHO, ROM1, RP1, RP9, RPE65, SAG, SEMA4A, SNRNP200, SPP2, TOPORS*
Autosomal recessive	65	RP22, RP29, RP32	*ABCA4, AGBL5, AHR, 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, SLC7A14, SPATA7, TRNT1, TTC8, TULP1, USH2A, ZNF408, ZNF513*
X-linked	6	RP6, RP24, RP34	*OFD1, RP2, RPGR*

Table 16.2 Details of RP genes

Gene	Location	Encoded protein	Associated diseases	References[a]
ABCA4	1p22.1	ATP-binding cassette transporter retinal	Recessive Stargardt disease, recessive MD, recessive RP, recessive fundus flavimaculatus, recessive CRD	[3]
ADIPOR1	1q32.1	Adiponectin receptor 1	Recessive RP, syndromic, Bardet-Biedl like, dominant RP	[4, 5]
AGBL5	2p23.3	ATP/GTP-binding protein-like 5	Recessive RP	[6]
AHR	7p21.1	Aryl hydrocarbon receptor	Recessive RP	[7]
ARHGEF18	19p13.2	Rho/Rac guanine nucleotide exchange factor 18	Recessive RP	[8]
ARL2BP	16q13.3	ADP-ribosylation factor-like 2 binding protein	Recessive RP	[9]
ARL3	10q24.32	ADP ribosylation factor like GTPase	Dominant RP	[10–12]
ARL6	3q11.2	ADP-ribosylation factor-like 6	Recessive BBS, recessive RP	[13]
BBS1	11q13	BBS1 protein	Recessive BBS, recessive RP	[14]
BBS2	16q13	BBS2 protein	Recessive BBS, recessive RP	[15]
BEST1	11q12.3	Bestrophin 1	Dominant MD, Best type; dominant vitreoretinochoroidopathy; recessive bestrophinopathy; recessive, dominant RP	[16]
C2orf71	2p23.2	Chromosome 2 open reading frame 71	Recessive RP	[17–20]
C8orf37	8q22.1	Chromosome 8 open reading frame 37	Recessive CRD, recessive RP with early macular involvement, recessive BBS	[21]
CA4	17q23.2	Carbonic anhydrase IV	Dominant RP	[22]
CERKL	2q31.3	Ceramide kinase-like protein	Recessive RP; recessive CRD with inner retinopathy	[23]
CLRN1	3q25.1	Clarin-1	Recessive USH, type 3; recessive RP	[24]
CNGA1	4p12	Rod cGMP-gated channel alpha subunit	Recessive RP	[25]
CNGB1	16q21	Rod cGMP-gated channel beta subunit	Recessive RP	[26]
CRB1	1q31.3	Crumbs homolog 1	Recessive RP with para-arteriolar preservation of the RPE; recessive RP, recessive LCA, dominant pigmented paravenous chorioretinal atrophy	[22]
CRX	19q13.32	Cone-rod otx-like photoreceptor homeobox transcription factor	Dominant CRD; recessive, dominant and de novo LCA; dominant RP	[27]
CYP4V2	4q35.2	Cytochrome P450 4V2	Recessive Bietti crystalline corneoretinal dystrophy, recessive RP	[28]
DHDDS	1p36.11	Dehydrodolichyl diphosphate synthetase	Recessive RP	[29, 30]
DHX38	16q22.2	DEAH (Asp-Glu-Ala-His) box polypeptide 38	Recessive RP, early onset with macular coloboma	[31]
EMC1	1p36.13	ER membrane protein complex subunit 1	Recessive RP	[32]
EYS	6q12	Eyes shut/spacemaker (*Drosophila*) homolog	Recessive RP	[33]
FAM161A	2p15	Family with sequence similarity 161 member A	Recessive RP	[34, 35]
FSCN2	17q25.3	Retinal fascin homolog 2, actin bundling protein	Dominant RP, dominant MD	[36]

(continued)

Table 16.2 (continued)

Gene	Location	Encoded protein	Associated diseases	References[a]
GPR125	4p15.2	G protein-coupled receptor 125	Recessive RP	[32]
GUCA1B	6p21.1	Guanylate cyclase activating protein 1B	Dominant RP, dominant MD	[37, 38]
HGSNAT	8p11.21-p11.1	Heparan-alpha-glucosaminide N-acetyltransferase	Recessive RP, recessive mucopolysaccharidosis	[39]
HK1	10q22.1	Hexokinase 1	Dominant RP, recessive nonspherocytic hemolytic anemia, recessive hereditary neuropathy (Russe type)	[40, 41]
IDH3B	20p13	NAD(+)-specific isocitrate dehydrogenase 3 beta	Recessive RP	[42]
IFT140	16p13.3	Intraflagellar transport 140 Chlamydomonas homolog protein	Recessive Mainzer-Saldino syndrome, recessive RP, recessive LCA	[43]
IFT172	2p33.3	Intraflagellar transport protein 172	Recessive BBS, recessive RP	[44]
IMPDH1	7q32.1	Inosine monophosphate dehydrogenase 1	Dominant RP, dominant LCA	[45]
IMPG2	3q12.3	Interphotoreceptor matrix proteoglycan 2	Recessive RP	[46]
KLHL7	7p15.3	Kelch-like 7 protein (*Drosophila*)	Dominant RP	[47]
KIAA1549	7q34	KIAA1549 protein	Recessive RP	[32, 48]
KIZ	20p11.23	Kizuna centrosomal protein	Recessive RP	[49]
LRAT	4q32.1	Lecithin retinol acyltransferase	Recessive RP, severe early-onset; recessive LCA	[50]
MAK	6p24.2	Male germ cell-associated kinase	Recessive RP	[51, 52]
MERTK	2q13	c-mer protooncogene receptor tyrosine kinase	Recessive RP; recessive RCD, early onset	[53]
MVK	12q24.11	Mevalonate kinase	Recessive RP, recessive mevalonic aciduria, recessive hyper-IgD syndrome	[54]
NEK2	1q32.3	NIMA (never in mitosis gene A)-related kinase 2	Recessive RP	[55]
NEUROD1	2q31.3	Neuronal differentiation protein 1	Recessive RP	[56]
NR2E3	15q23	Nuclear receptor subfamily 2 group E3	Recessive enhanced S-cone syndrome; recessive, dominant RP; recessive Goldman-Favre syndrome; combined dominant and recessive retinopathy	[57, 58]
NRL	14q11.2	Neural retina leucine zipper	Dominant, recessive RP	[59, 60]
OFD1	Xp22.2	Oral-facial-digital syndrome 1 protein	Jobert syndrome; orofaciodigital syndrome 1, Simpson-Golabi-Behmel syndrome 2; X-linked RP, severe	[61]
PDE6A	5q33.1	cGMP phosphodiesterase alpha subunit	Recessive RP	[62]
PDE6B	4p16.3	Rod cGMP phosphodiesterase beta subunit	Recessive RP; dominant CSNB	[63]
PDE6G	17q25.3	Phosphodiesterase 6G cGMP-specific rod gamma	Recessive RP	[64]

Table 16.2 (continued)

Gene	Location	Encoded protein	Associated diseases	References[a]
POMGNT1	1p34.1	Protein O-linked acetylglucosaminyltransferase 1 (beta 1,2-)	Recessive RP	[65]
PRCD	17q25.1	Progressive rod-cone degeneration protein	Recessive RP	[66]
PROM1	4p15.32	Prominin 1	Recessive RP with macular degeneration; dominant Stargardt-like MD; dominant MD, bull's-eye; dominant CRD	[67]
PRPF3	1q21.2	Pre-mRNA processing factor 3	Dominant RP	[68]
PRPF4	9q32	Pre-mRNA processing factor 4	Dominant RP	[69, 70]
PRPF6	20q13.33	Pre-mRNA processing factor 6	Dominant RP	[71]
PRPF8	17p13.3	Pre-mRNA processing factor 8	Dominant RP	[72]
PRPF31	19q13.42	Pre-mRNA processing factor 31	Dominant RP	[73]
PRPH2	6p21.1	Peripherin 2	Dominant RP, dominant MD, digenic RP with ROM1, dominant adult vitelliform MD, dominant CRD, dominant central areolar choroidal dystrophy, recessive LCA	[74–76]
RBP3	10q11.22	Retinol binding protein 3, interstitial	Recessive RP	[77]
RDH12	14q24.1	Retinol dehydrogenase 12	Recessive LCA, dominant RP	[78]
REEP6	19p13.3	Receptor expression enhancer protein 6	Recessive RP	[79]
RGR	10q23.1	RPE-retinal G protein-coupled receptor	Recessive RP, dominant choroidal sclerosis	[80]
RHO	3q22.1	Rhodopsin	Dominant, recessive RP; dominant CSNB	[81, 82]
RLBP1	15q26.1	Retinaldehyde-binding protein 1	Recessive RP, recessive Bothnia dystrophy, recessive retinitis punctata albescens, recessive Newfoundland RCD	[83]
ROM1	11q12.3	Retinal outer segment membrane protein 1	Dominant RP, digenic RP with *PRPH2*	[76, 84]
RP1	8q12.1	RP1 protein	Dominant, recessive RP	[85, 86]
RP1L1	8p23.1	RP1-like protein 1	Dominant occult MD, recessive RP	[87]
RP2	Xp11.23	RP 2 (X-linked)	X-linked RP; X-linked RP, dominant	[88]
RP9	7p14.3	RP9 protein or PIM1-kinase associated protein 1	Dominant RP	[89]
RPE65	1p31.2	Retinal pigment epithelium-specific 65 kD protein	Recessive LCA, recessive RP, dominant RP with choroidal involvement	[90]
RPGR	Xp11.4	RP GTPase regulator	X-linked RP, recessive, dominant; X-linked cone dystrophy; X-linked atrophic MD, recessive	[91, 92]
SAMD11	1p36.33	Sterile alpha motif domain containing 11 protein	Recessive RP	[93]
SAG	2q37.1	Arrestin (s-antigen)	Recessive Oguchi disease; recessive, dominant RP	[94, 95]
SEMA4A	1q22	Semaphorin 4A	Dominant RP, dominant CRD	[96, 97]
SLC7A14	3q26.2	Solute carrier family 7 member 14	Recessive RP	[98]

(continued)

Table 16.2 (continued)

Gene	Location	Encoded protein	Associated diseases	References[a]
SNRNP200	2q11.2	Small nuclear ribonucleoprotein 200kDa (U5)	Dominant RP	[99, 100]
SPATA7	14q31.3	Spermatogenesis associated protein 7	Recessive LCA; recessive RP, juvenile	[101]
SPP2	2q37.1	Secreted phosphoprotein 2	Dominant RP	[102]
TOPORS	9p21.1	Topoisomerase I binding arginine/serine rich protein	Dominant RP	[103]
TRNT1	3p26.2	CCA adding tRNA nucleotidyl transferase 1	Recessive RP with erythrocytic microcytosis; recessive RP, non-syndromic	[104]
TTC8	14q32.11	Tetratricopeptide repeat domain 8	Recessive BBS, recessive RP	[105]
TULP1	6p21.31	Tubby-like protein 1	Recessive RP, recessive LCA	[106–108]
USH2A	1q41	Usherin	Recessive USH, type 2a; recessive RP	[109]
ZNF408	11p11.2	Zinc finger protein 408	Dominant familial exudative vitreoretinopathy, recessive RP with vitreal alterations	[110, 111]
ZNF513	2p23.3	Zinc finger protein 513	Recessive RP	[112, 113]

Abbreviations: MD, macular dystrophy; RP, retinitis pigmentosa; CRD, cone-rod dystrophy; BBS, Bardet-Biedl syndrome; USH, Usher syndrome; LCA, Leber congenital amaurosis; RCD, rod-cone dystrophy; CSNB, congenital stationary night blindness

[a]Only references reporting association between the gene and RP are included

activation of the cGMP phosphodiesterase [120, 121]. In addition, guanylate cyclase-activating proteins (encoded by the *GUCA1A*, *GUCA1B*, and *GUCA1C* genes) activate guanylate cyclase (encoded by the *GUCY2D* gene), which increases the concentration of cGMP in photoreceptor plasma to normal levels [122, 123].

In cone photoreceptors, most molecules involved in the rod phototransduction have a homolog with similar biological functions. However, unlike rhodopsin in rod cells, cone cells express three opsins specific to distinct wavelength and have much faster kinetics, which leads to a shorter recovery phase [124]. Concentration of the GTPase-accelerating protein complex is ten times higher in cones compared to rods.

16.4 The Visual Cycle

The visual cycle is a complex process that regenerates 11-*cis*-retinal from all-*trans*-retinal produced in the phototransduction cascade. The canonical visual cycle in rod photoreceptors happens spontaneously upon the phototransduction, when all-*trans*-retinal dissociates from the visual pigment, enters the lumen of the outer segment discs, and converts phosphatidylethanolamine to N-retinylidene-phosphatidylethanolamine [125]. All-*trans*-retinal is then transported into the photoreceptor cytoplasm through the flippase activity of the adenosine triphosphate (ATP)-binding cassette transporter ABCR (encoded by the *ABCA4* gene), to form all-*trans*-retinol by the enzyme all-*trans*-retinal dehydrogenase (encoded by the *RDH8*, *RDH12*, and *RDH14* genes) [126, 127]. All-*trans*-retinol is then released to the subretinal space to bind to the cellular retinol-binding protein (IRBP, encoded by the *RBP3* gene) [128], and further moves to the RPE cytoplasm, where it binds to the cellular retinol-binding protein (encoded by the *CRBP1* gene) and is re-isomerized through a cascade involving lecithin-retinol acyltransferase (LRAT), RPE65, retinal G protein-coupled receptor (RGR), and 11-*cis*-retinol dehydrogenase (encoded by the *RDH5* and *RDH11* genes) [124, 129, 130]. The generated 11-*cis*-retinal is then transferred into the inter-photoreceptor matrix by cellular retinaldehyde-binding protein (CRALBP, encoded by the

RLBP1 gene), and is transferred back into the photoreceptor's cytoplasm by IRBP to bind to opsin to generate a new rhodopsin molecule.

Other than the above-mentioned canonical visual cycle, cones have another noncanonical visual cycle, which regenerates 11-*cis*-retinal at a 20-fold faster rate [131, 132]. This noncanonical visual cycle happens in the cone outer segments and Müller cells, and is triggered upon photobleaching of cone-specific opsin. All-*trans*-retinal is released into the cone plasma, and is reduced to all-*trans*-retinol by retinol dehydrogenases (encoded by the *RDH8* and *RDH14* genes) and the cone-specific enzyme retSDR1 (encoded by the *DHRS3* gene) [133]. The generated all-*trans*-retinol then binds to IRBP and moves to Müller cells, where it is catalyzed by dihydroceramide desaturase-1 (DES1, encoded by the *DEGS1* gene) to produce the isomerized 11-*cis*-retinol, 9-*cis*-retinol, and 13-*cis*-retinol [132, 134]. Since the isomerization catalyzed by DES1 is reversible [134], the generated isomerized 11-*cis*-retinol has the potential to be re-isomerized, which can be prevented when bound to the CRALBP [135]. 11-*cis*-retinol is then transported into the interphotoreceptor matrix to bind IRBP, and subsequently moved to the cone outer segment [136], where it is oxidized to form 11-*cis*-retinal. The 11-*cis*-retinal then binds to cone opsins and form a new pigment molecule.

16.5 Ciliary Structure and Transport

Cilia are slender protuberances that are projected from the surface of most mammalian cells [137]. Cilia can be divided into motile forms and primary forms. In rod photoreceptor cells, the apical outer segment is connected to its basal body with a specialized nonmotile cilium [138]. Since the outer segment lacks biosynthetic machinery, all its components are synthesized and partially pre-assembled in the inner segment and then transferred to the outer segments through the connecting cilium, which is facilitated by the intraflagellar transport (IFT). By far, mutations in more than 30 ciliary genes have been found associated with non-syndromic retinal diseases [139], and ciliary genes linked to non-syndromic RP encode proteins that are involved in various aspects of ciliary transport. For example, IFT is medicated by the IFT proteins, including IFT140 (encoded by the *IFT140* gene) and IFT172 (encoded by the *IFT172* gene), which form two complexes, and bind and transport ciliary cargo [140]. The BBSome, a complex of eight Bardet-Biedl syndrome (BBS) proteins (BBS1, BBS2, BBS4, BBS5, BBS7, TTC8, BBS9, and BBS18) [141], serves as an adaptor between the IFT complex and the ciliary cargo [141]. Mutations in most BBSome components cause BBS [142], while four of BBSome subunits, including BBS1 (encoded by the *BBS1* gene), BBS2 (encoded by the *BBS2* gene), TTC8 (encoded by the *TTC8* gene), and BBS9 (encoded by the *BBS9* gene), together with ARL6 (encoded by the *ARL6* gene), which recruits BBSome complex to the membrane, are linked to non-syndromic RP [14, 15, 105, 143–148].

In addition, ARL3 (encoded by the *ARL3* gene) and RP2 (encoded by the *RP2* gene) mediate the localization of motor units at the ciliary tip [149]. Moreover, RPGR (encoded by the *RPGR* gene, which accounts for 70–90% of X-linked RP cases [150]) participates in assembly of the transition zone and control of the cilium gating function. RPGR binds to another two ciliary proteins, RPGR interacting protein 1 (RPGRIP1) and spermatogenesis associated protein 7 (SPATA7, encoded by the *SPATA7* gene), in the connecting cilium to form the RPGR–RPGRIP1–SPATA7 complex, which plays crucial roles in transporting specific opsins [151]. Meanwhile, RPGR could also interact with several other ciliary proteins associated with retinal diseases, like centrosomal protein 290 (encoded by the *CEP290* gene), nephrocystin 1 (encoded by the *NPHP1* gene), and nephrocystin 4 (encoded by the *NPHP4* gene) [124, 152].

Compliance with Ethical Requirements Xue Chen and Chen Zhao declare that they have no conflict of interest. No human or animal studies were performed by the authors for this chapter.

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Primary Congenital Glaucoma Genetics: The Experience in Brazil

17

Mônica Barbosa de Melo,
José Paulo Cabral de Vasconcellos,
and Vital Paulino Costa

Abstract

Primary congenital glaucoma (PCG) is the most prevalent form among childhood glaucomas, with an incidence varying between 1:1250 and 1:30,000. The majority of PCG cases are sporadic and families have been reported with an autosomal recessive inheritance pattern and variable penetrance. Genetic heterogeneity has been observed in PCG. Five loci have been identified (*GLC3A–GLC3E*) and, among these loci, variants in three genes have been associated with PCG.

In Brazil, to date the genetic profile of PCG is restricted to the evaluation of the *CYP1B1* gene. Brazil is a country characterized by a highly admixed population and low frequency of consanguineous marriages. Most studies have been conducted in the Southeast and report a frequency of disease-associated variants ranging from 23.5 to 50.0%, with most variants present in compound heterozygosity and some variants still unique to this population. An association between variants in the *CYP1B1* gene and poor prognosis has also been observed, reinforcing the importance of investigating this gene in Brazilian PCG patients.

Keywords

Primary congenital glaucoma · Genetics
CYP1B1 · Gene · Brazil

17.1 Introduction

Glaucoma comprises several conditions that affect the optic nerve leading to structural changes characterized by loss of retinal nerve fiber layer and optic disc cupping with corresponding visual field defects. The pathophysiology of glaucoma involves many combined mechanisms and metabolic pathways, but the main one includes the increase of intraocular pressure (IOP). IOP is determined by the equilibrium of aqueous humor production at the ciliary body and its outflow through the trabecular meshwork (conventional outflow) and ciliary muscle fibers (uveoscleral outflow). In general terms, the glaucomas are classified according to their etiology, anterior chamber anatomy, and age of onset. In respect to etiology, glaucoma can be divided into primary (with no identified cause) and secondary to an

M. Barbosa de Melo (✉)
Center for Molecular Biology and Genetic Engineering, University of Campinas, Campinas, São Paulo, Brazil

J. P. Cabral de Vasconcellos · V. Paulino Costa
Department of Ophthalmology and Otorhinolaryngology, School of Medical Sciences, University of Campinas, Campinas, São Paulo, Brazil

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ocular or systemic condition. Glaucomas can also be classified according to the anatomic characteristic of the anterior chamber angle (open angle or closed angle), and finally depending on the age of onset (adult or pediatric) [1–3].

In 2013, an international consortium of glaucoma specialists named Childhood Glaucoma Research Network proposed a classification of pediatric glaucomas based on clinical aspects and in the context of clinical and ocular features in which the diagnosis was made. Therefore, both primary congenital glaucoma (PCG) and juvenile open-angle glaucoma are classified as primary childhood glaucomas, since none is associated with acquired ocular anomalies, systemic disease, or syndromes [4]. Among the pediatric glaucomas, PCG is the most prevalent, with an incidence varying between 1:1250 and 1:30,000, depending on the population that is investigated. In general, the more inbred the population, the higher the incidence [5].

17.2 Epidemiology

In Brazil, there is no robust study indicating the prevalence/incidence of PCG. Available data comes from studies performed at University Hospitals. One of these studies evaluated 72 children with pediatric glaucoma, showing 61.5% with PCG and 38.5% with secondary congenital glaucoma [6].

Other studies have evaluated the causes of visual impairment in children at low vision services in Brazilian public hospitals. It is important to emphasize that Brazil is a country with a continental dimension, with the North region being less developed than the South region. These socioeconomic characteristics lead to different frequencies in causes of visual impairment in children: infections tend to be the leading cause in the North, whereas retinopathy of prematurity is more frequent in the South. In a study that evaluated children from the rural area, the main causes of low vision were congenital cataract and toxoplasmosis (14.0%) followed by congenital glaucoma [7]. Another study, performed at the University of São Paulo, the most developed state in Brazil, involving 3210 cases, also showed macular toxoplasmosis as the first cause of visual impairment in children with low vision (20.7%), followed by retinal dystrophies (12.2%), retinopathy of prematurity (11.8%), ocular malformations (11.6%), and congenital glaucoma (10.8%) [8].

A recent update about the causes of childhood blindness worldwide situates glaucoma as the third leading cause in the Americas. Interestingly, glaucoma does not appear as one of the three main causes of blindness in the other regions of the world (Africa, Eastern Mediterranean, Europe, Southeast Asia, and Western Pacific) [9].

17.3 Mechanisms and Clinical Features

The presumed mechanism in PCG development is related to a dysfunction in the outflow system, particularly at the conventional outflow. Trabecular meshwork and Schlemm canal are both structures derived from the neural crest and mesodermal lineage. During the embryonic period, these tissues do not complete their maturation, which results in decreased outflow and IOP rise. The effect of increased IOP in ocular structures go beyond glaucomatous optic nerve damage, including ocular globe enlargement (buphthalmos), increase in corneal diameter (megalocornea), and breaks in the corneal endothelium (Haab striae). These ocular alterations lead to a classical clinical triad of GCP symptoms including epiphora, blepharospasm, and photophobia [10, 11].

The Childhood Glaucoma Research Network has introduced a classification guide to PCG diagnosis defined as the presence of two or more of the following criteria: IOP greater than 21 mmHg, optic disc cupping, corneal abnormalities related to PCG, ocular enlargement evaluated by axial length or progressive myopia, and visual field defect consistent with glaucoma [4]. The treatment of PCG aims at reducing IOP and controlling amblyopia. In the vast majority

of cases, IOP control requires initially an angular surgical procedure in order to improve aqueous humor outflow. PCG is mostly bilateral and asymmetrical with no sex preference in familial cases, but with higher male prevalence in sporadic cases [3, 10].

17.4 Genetic Aspects

The majority of PCG cases are sporadic and families have been reported with an autosomal recessive inheritance pattern and variable penetrance. Since the observation of inheritance patterns and the advances of molecular biology tools, several families have been evaluated, leading to the identification of genes associated with PCG. Genetic heterogeneity has been observed in PCG, what means that the same clinical phenotype results from variants in different loci/genes or that different patients, with the same genetic disease, present with different alterations in the same gene [3, 11]. Five loci have been identified (*GLC3A*–*GLC3E*) and, among these loci, variants in three genes have been associated with PCG [12].

Sarfarazi et al. identified the first gene, cytochrome P4501B1 (*CYP1B1*), located on chromosome 2p21 linked to *GLC3A* locus in Pakistani families [13, 14]. *CYP1B1* belongs to the cytochrome P450 family of membrane-bound oxidase enzymes and codes for P4501B1, a monooxygenase probably involved in the metabolism of a variety of substrates, including steroids and retinoids. Different from other P450 proteins, CYP1B1 is highly expressed outside the liver, particularly in tissues responsible for IOP homeostasis: trabecular meshwork and ciliary body [15, 16].

The mechanism through which disease-associated variants cause PCG is not completely understood. It is suggested that the enzyme codified by the gene would participate in metabolic pathways involved in the development of the anterior chamber, particularly, in the formation of the trabecular meshwork, via degradation of certain metabolites, as well as in the clearance of reactive oxygen species. Hence, variants in the *CYP1B1* gene could compromise the development and differentiation of this tissue, leading to IOP elevation and consequent optic nerve damage [17, 18].

The *CYP1B1* gene consists of three exons, one non-coding, and two introns [19]. More than 150 variants associated with PCG have been described, according to the "The Human Gene Mutation Database" (HGMD) [20]. The distribution of mutations can vary worldwide, from 14 to 30% in North American and European populations, from 15 to 20% in Chinese and Japanese populations and from 90 to 100% in Saudi Arabians and Slovakian Gypsies [3, 21–26]. The type of disease-associated variants can also be more frequent in certain populations. For example, E387K seems to be a founder variant in Slovakian Gypsies, G61E is a founder mutation in the Middle Eastern population and R390H is common among Asian populations [27].

Following the identification of *GLC3A* locus and its corresponding *CYP1B1* gene, two other loci, *GLC3B* and *GLC3C*, located on 1p36 and 14q24, respectively, were reported, but no PCG-associated variants have been identified [28, 29].

The *GLC3D* locus is also located on 14q24 and encompasses the latent transforming growth factor beta-binding protein 2 (*LTBP2* gene). *LTBP2* gene was identified through linkage analysis in Pakistani and Iranian PCG families presenting with autosomal recessive inheritance pattern. This gene encodes an extracellular matrix protein expressed in tissues with high concentration of elastic fibers with putative function in elastin microfibril assembly and cell adhesion. Its expression in ocular tissues such as the trabecular meshwork and ciliary body, as well as its role in anterior chamber development, make disease-causing variants in *LTBP2* gene a reasonable cause of PCG. Unlike the worldwide distribution of *CYP1B1* gene variants, *LTBP2* alterations have been reported in few populational groups [11, 30].

The most recently identified locus is *GLC3E*, which contains the tunica interna endothelial

cell kinase (*TEK*) gene. This gene was not found in a family-based linkage study, but in transgenic mice that harbored deletions in *TEK* gene or in both major angiopoietin ligands. These transgenic mice had a developmental loss of Schlemm's canal, resulting in IOP rise and ganglion cell loss compatible with a PCG phenotype. These findings led to a candidate gene approach involving 189 unrelated PCG patients, of whom ten presented heterozygous disease-causing variants in the human *TEK* gene [31].

17.4.1 *CYP1B1* Gene Screening in Brazilian PCG Patients

The first study that described the analysis of the *CYP1B1* gene in PCG Brazilian patients was a collaboration between two Brazilian Universities from the state of São Paulo and the group directed by Dr. Mansoor Sarfarazi [32]. Fifty-two patients were evaluated through single-strand conformation polymorphism and Sanger sequencing: 51.9% presented positive family history, consanguinity was reported by 26.9 and 84.6% had bilateral PCG. Fifty percent of the patients showed disease-associated variants. The majority of them were present in familial versus sporadic cases (55.6 versus 41.7%) and in bilateral versus unilateral disease (55.8 versus 12.5%). Homozygosity was reported in 57.7% of the cases, heterozygosity in 15.4%, and compound heterozygosity in 26.9%. Eleven different mutations have been identified, four of them described for the first time (g.3860C>T, g.4340delG, g.8165C>G and g.8214_8215delAG). The 4340delG variant was present in 46.0% (12/26) of PCG cases positive for *CYP1B1* alterations (nine homozygotes, two compound heterozygotes, and one heterozygote), associated with a severe phenotype, coursing with early onset (91.7% of the cases in the first month), worse clinical prognosis (all bilateral cases, high IOP in 11/12 cases), and limited response to surgical treatment. The most frequent haplotype observed among Brazilian patients was 5′-CCGGTA-3′, which was associated with at least seven mutations and probably with 4340delG.

In a report from Hollander et al., a deeper genotype–phenotype correlation for *CYP1B1* variants was performed. The trabeculectomy specimens from patients harboring variants were analyzed showing different extent of goniodysgenesis dependent on the genotype. One of the patients was a compound heterozygote for 4340delG and C209R. This patient showed severe goniodysgenesis, with agenesis of the Schelemm's canal [33].

In a joint study of Brazilian and American families, three variants in the *CYP1B1* gene have been identified in two Brazilian families (g.8037_8046dupTCATGCCACC in homozygosity, g.8182delG, and p.Glu387Lys in compound heterozygosity) by Sanger sequencing. In both pedigrees, the disease presented with corneal edema, early onset and high IOP. The same variants were reported in one of the American families (g.8037_8046dupTCATGCCACC and p.Glu387Lys), who also showed the p.268delSNF variant. Patients who harbor these variants shared a common haplotype, indicating a common founder between these two populations [34].

Della Paolera et al. conducted another study with 30 patients from the state of São Paulo [35]. PCG was bilateral in 66.7% of the cases and unilateral in 33.3%. All patients underwent a surgical procedure before the age of 3 months and all cases were sporadic, with no consanguineous marriage being reported. Thirty percent of the patients (9/30) presented *CYP1B1* variants, detected by Sanger sequencing and ten different mutations were described, two of them for the first time (4523delC and L378Q). Four of the patients presented variants in compound heterozygosis, two in homozygosis and in three patients only one mutant allele was identified. Prognosis was worse in patients who harbored alterations in the *CYP1B1* gene: mean IOP at diagnosis was higher, more surgical procedures were necessary for IOP control (the risk of patients positive for *CYP1B1* alterations to undergo more than one surgical procedure was nine times greater than the negative ones), and all patients had bilateral glaucoma.

Different from the first study conducted in the population from São Paulo state, the 4340delG variant was present in only two (6.7%) patients in heterozygosis. Two patients presented one of the new variants, 4635delT, in homozygosis. Both patients had severe bilateral disease, with two to three surgeries in each eye to control IOP and important visual function impairment. The 4523delC and L378Q alterations were present in compound heterozygosity in three members from the same family, all with high IOP at diagnosis, difficult surgical control and poor visual function.

Few years ago, a study involving Indian and Brazilian GCP patients evaluated 301 and 150 patients, respectively [36]. This study encompassed Brazilian patients from two previous studies as well as 68 new cases [32, 35]. A frequency of approximately 44.0% disease-associated variants in the *CYP1B1* gene has been reported in both populations. Despite the similar frequency, variants in homozygosis were more frequent in the Indian cohort (24.2% versus 16.7%) while compound heterozygosis was more frequent in the Brazilian cohort (12.7% versus 6.0%), which is probably due to the higher rate of consanguineous marriages among Indians. Both populations exhibited significant allelic heterogeneity. Thirty-nine variants were reported in Indian patients, while 17 in Brazilian patients. Most of these variants were population specific. Thirty-three were present only in Indian patients, while 11 were reported only in Brazilian patients. Six variants were shared between both groups (g.8037_8046dup10, g.8214_8215delAG, p.R368H, p.P437L, p.A443G, and p.S476P). The most prevalent alterations were R368H in India and 4340delG in Brazil. The R368H was observed in only three Brazilian patients (in homozygosity and compound heterozygosity) and the 4340delG was observed only in the Brazilian cohort. Regarding haplotype distribution, as observed in previous studies, the 5′-CCGGTA-3′ was a risk haplotype, associated with most variants.

In the group of Brazilian patients, 44.0% of the patients showed *CYP1B1* disease-associated variants. Although not statistically significant, age of onset was lower in the group positive for *CYP1B1* alterations. This group also showed higher frequency of family history and consanguinity. When all Brazilian PCG samples were evaluated (52 from the first study, 30 from the second study, and 68 from this study) no association was observed between alterations in the *CYP1B1* gene, IOP, and corneal diameter. In this report, the number of surgeries and number of affected eyes were not evaluated in relation to *CYP1B1* changes.

Another example of a patient from the Southeast Brazil is a 2-month-old male infant with bilateral PCG who was screened for variants in the *CYP1B1* gene. Glaucoma was diagnosed when he was less than 1 month old. The patient presented IOP of 26 mmHg in the right eye and 28 mmHg in the left eye, axial length of 21.49 mm in the right eye and 22.20 mm in the left eye, as well as buphthalmos, megalocornea, and corneal edema. The child has been submitted to four surgeries to control IOP. The *CYP1B1* gene screening showed the presence of two different variants (compound heterozygosity): p.E387K inherited from the father and p.R444Ter, inherited from the mother. As far as we know the R444Ter variant is being described for the first time in Brazil. The parents had no glaucoma or family history of glaucoma and no consanguinity (Fig. 17.1, data not published).

Recently, a study was conducted by Coêlho and collaborators who evaluated 17 PCG patients from an ethnically diverse population from the Northeast Brazil through next-generation sequencing [37]. Most of the patients had bilateral glaucoma (88.2%), the age at diagnosis ranged from 0 to 9 years and in 52.9% of the patients at least two surgical procedures were required. The late diagnosis reflects the poor health care quality in the Northeast compared to the Southeast region of Brazil. Disease-associated variants were present in 23.5% of the patients, three compound heterozygotes and one homozygote, and five different variants were reported, two of which were described for the first time in Brazilian patients (p.G61E and p.Y81N). No genotype–phenotype correlation was observed.

All variants that have been reported in Brazilian PCG patients are depicted in Table 17.1.

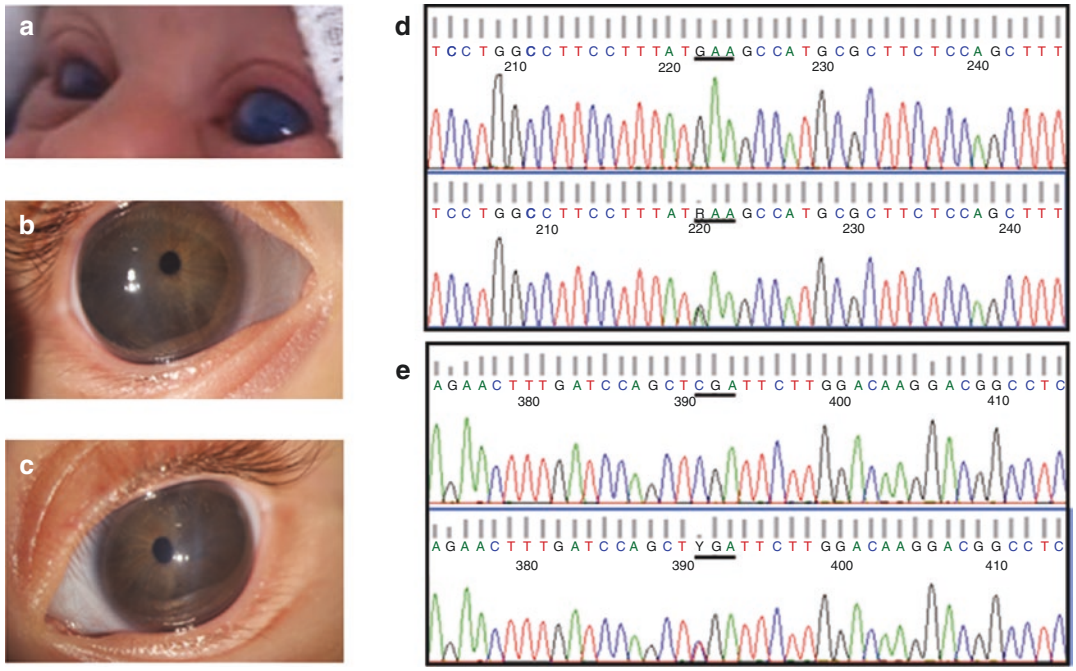

Fig. 17.1 Primary congenital glaucoma patient harboring variants in the *CYP1B1* gene. (**a**) Photograph of the patient at diagnosis showing corneal edema and buphthalmos. (**b**) Left eye after surgeries for IOP reduction. (**c**) Right eye after surgeries for IOP reduction. (**d**) Chromatogram showing the p.E387K variant (GAA-AAA) in heterozygosis. (**e**) Chromatogram showing the p.R444Ter variant (CGA-TGA) in heterozygosis. Photographs are courtesy of Dr. Christiane Rolim de Moura from Federal University of São Paulo

Table 17.1 Distribution of *CYP1B1* mutations associated with PCG observed in Brazilian cohorts

Genomic DNA position	Amino acid change	Allele frequencies (%)	Origin
g.3860C>T	p.Q19Ter	1.14	Brazil [32, 35–37], South Korea [42]
g.3976 G>A	p.W57Ter	1.70	Brazil [32, 36], Australia [43], USA [26], Germany [44], France [45], Hipanic origin [46]
g.3987 G>A	p.G61E	0.28	Brazil [37], Saudi Arabia [21, 23, 47], Iran [48, 49], USA [44], Morocco [50], India [51, 52], Turkey [53], Spain [54], Ecuador [55]
g.4046 T>A	p.Y81N	0.28	Brazil [37], Pakistan [56], Germany [44], Spain [54]
g.4340delG	Frameshift	10.51	Brazil [32, 35, 36], Morocco [50], USA, Hispanic origin [33], North Africa [45]
g.4523delC	Frameshift	0.28	Brazil [35]
g.4635delT	Frameshift	1.70	Brazil [35, 36], Mexico [57]
g.7901_7913delGAGTGCAGGCAGA	Frameshift	3.40	Brazil [32, 35, 36], Turkey [14, 46, 58], France [45], Saudi Arabia [47], USA [26, 44], Russia, Germany, Switzerland [44], Canada [59], Spain [54]
g.7940G>A	p.R368H	0.85	Brazil [32, 36], Saudi Arabia [23], Iran [48, 49], India [36, 51, 52], Turkey [53], Australia [43], USA [44], Pakistan [56], South Korea [42], Germany [44]

Table 17.1 (continued)

Genomic DNA position	Amino acid change	Allele frequencies (%)	Origin
g.7970 T>A	p.L378Q	0.57	Brazil [35, 36]
g.7996 G>A	p.E387K	1.42	Brazil [32, 34–36], Romany [22], France [45], Canada [59], USA [26, 34, 44], Australia [43], Hispanic origin [46]
g.8035 C>T	p.P400S	0.28	Brazil [36], Australia [43], Spain [54]
g.8037_8046dupTCATGCCACC	Frameshift	5.11	Brazil [32, 35–37], France [45], India [36, 51], Turkey [46, 53], USA [44, 46], UK [46], Pakistan [56], Spain [54]
g.8147C>T	p.P437L	1.70	Brazil [32, 36, 37], Turkey [46], India [36, 51], Saudi Arabia [47], Spain [54]
g.8165 C>G	p.A443G	1.14	Brazil [32, 35, 36], Saudi Arabia [47], Ethiopia [60], Lebanon [58], USA [26], India [36]
g.8168 G>A	p.R444Q	0.28	Brazil [36], Japan [61], South Korea [42], Australia [43], France [45]
g.8182delG	Frameshift	2.27	Brazil [32, 35], USA [46], Portugal [45]
g.8214_8215delAG	Frameshift	0.85	Brazil [32, 35, 36], India [36]
g. 8263 T>C	p.S476P	0.28	Brazil, India [36]

As previously reported, the Brazilian population is highly admixed and heterogeneous. It is the result of several immigration events accompanied by the miscegenation of three major ancestral roots: Amerindians, Europeans, and Africans. Genetic composition varies from region to region, but it has been shown that the urban population is more uniform than previously thought. For autosomal markers, the proportion of European, African, and Amerindian ancestries was estimated between 70 and 77%, 13 and 19%, and 9 and 10%, respectively [38–40]. Accordingly, the study by Rolim et al. evaluated ancestry markers in PCG patients from the State of Minas Gerais and reported that the proportion of Europeans, Africans, and Amerindians ranged from 74 to 83%, 11 to 18%, and 4 to 9%, respectively. The authors demonstrated that African ancestry was more frequent in PCG cases than in controls (although with no statistical significance) and that it was associated with a higher number of surgeries to control IOP, suggesting that it might act as risk factor for the disease when in high proportion [41].

17.5 Summary

The studies evaluating the participation of the *CYP1B1* gene as causative for PCG in Brazil have shown a frequency of disease-associated variants ranging from 23.5 to 50.0%. This important contribution strongly suggests that this gene is worth being tested in Brazilian PCG patients. Most of the patients present compound heterozygosity in their genotype, reinforcing the admixture profile of the Brazilian population.

The most frequent disease-associated variants in Brazil are g.4340delG, followed by g.8037_8046dupTCATGCCACC, and g.7901_7913delGAGTGCAGGCAGA. It is important to notice that only one study was performed in the Northeast region, with only 17 PCG patients included. This was enough to identify two new variants in Brazil, which emphasizes the need for additional studies in all regions of Brazil, in order to obtain a more realistic representation of PCG in this population.

Twenty years after the identification of *CPYP1B1* gene, two variants remain exclusively

identified in Brazil: g.4523delC and L378Q. The other variants are shared with several population groups, but four were reported in only one other country: South Korea (p.Q19Ter), Mexico (g.4635delT), and India (g.8214_8215delAG and p.S476P). It would be interesting to evaluate if these four disease-associated variants are originated from a common founder or if they are de novo events.

Brazil can contribute in the understanding of the genetic basis of PCG by searching for new genes using family-based approach, as well as investigating the genes recently associated with PCG, *LTBP2*, and *TEK*. The latter seems to be more promising, since their disease-associated variants appear to be more spread in different populations than *LTBP2* variants. Finally, it is important that more collaborative studies are made to better reveal the genetic basis of PCG and to establish genotype–phenotype correlations applicable in precision medicine. For example, if *TEK* alterations are associated with the absence of Schlemm's canal, the primary angle surgery might not be the ideal surgical treatment option.

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Glaucoma Genetics in Pakistan

18

Humaira Ayub, Maleeha Azam, Shazia Micheal, Muhammad Imran Khan, Farah Akhtar, Chiea Chuen Khor, Tin Aung, Anneke den Hollander, and Raheel Qamar

Abstract

Glaucoma, a multifactorial ocular disease, is clinically and genetically heterogeneous. It is the second leading cause of blindness in elderly population worldwide. Because of the complex nature of glaucoma, the genetic spectrum has not been established globally. In Pakistan, both the familial and the sporadic forms of the disease are common, which is attributed to higher percentage of consanguinity in the Pakistani population. Till the year 2008, there were no reports from Pakistan about the genetic factors causing glaucoma. In order to identify the glaucoma genetic spectrum in the Pakistani population, we genetically screened individuals with glaucoma that included the common clinical subclasses; primary congenital glaucoma (PCG), primary open angle glaucoma (POAG), primary angle closure glaucoma (PACG), and pseudoexfoliation glaucoma (PEXG). We conducted linkage analysis of the glaucoma families,

H. Ayub
Department of Biomedical Sciences, Pak-Austria Fachhochschule: Institute of Applied Sciences and Technology Mang, Haripur, Pakistan

M. Azam
Department of Biosciences, COMSATS University Islamabad, Islamabad Campus, Islamabad, Pakistan

S. Micheal
Department of Ophthalmology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

M. Imran Khan
Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

F. Akhtar
Al-Shifa Trust Eye Hospital, Rawalpindi, Pakistan

C. C. Khor
Division of Human Genetics, Genome Institute of Singapore, Singapore, Singapore
e-mail: khorcc@gis.a-star.edu.sg

T. Aung
Singapore Eye Research Institute (SERI) & Singapore National Eye Center (SNEC), Singapore, Singapore

Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore
e-mail: aung.tin@singhealth.com.sg

A. den Hollander
Department of Ophthalmology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands

Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands
e-mail: A.denHollander@ohk.umcn.nl

R. Qamar (✉)
Department of Biosciences, COMSATS University Islamabad, Islamabad Campus, Islamabad, Pakistan

ICESCO, Rabat, Morocco

Pakistan Academy of Sciences, Islamabad, Pakistan

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case-control association analysis of the sporadic glaucoma cases using previously reported single nucleotide polymorphisms (SNPs), and also carried out genome wide association studies (GWAS). These studies have allowed us to discover novel glaucoma causing genes and risk-associated SNPs in the Pakistani population. The identification of novel glaucoma genes reveals novel molecular mechanisms involved in glaucoma pathogenesis. However, the clinical heterogeneity in the Pakistani glaucoma population suggests the need for further exploration of the molecular/genetic causes of the disease.

Keywords

Glaucoma · GWAS · SNPs · *CYP1B1* · Novel genes/loci

18.1 Introduction

Glaucoma is a group of neurodegenerative ocular diseases, which is caused by optic nerve damage either in one eye or both, leading to visual field defect and eventually blindness.

Glaucoma is a multifactorial disease, caused by interplay of genetic and environmental factors. The complex nature of the disease is either due to heterogeneity where different genes result in the same clinical subtype or the same genes lead to variable clinical conditions. Phenocopies have also been observed that are caused by environmental factors. The presence of modifier element also makes the condition complex where mutation carriers remain asymptomatic. Deviation from the Mendelian mode of inheritance is common in glaucoma [1–4]. Due to all these different factors analyzing glaucoma genetically is difficult, however, familial clustering as well as association studies have been helpful in identification of pathogenic mutations as well as rare and common polymorphic genetic variants that have shown higher percentages in affected individuals as compared to healthy (unaffected) control population [5]. Having a multifaceted etiology, certain genes are pertinent to glaucoma progression globally as well as in a population-specific manner. In Pakistan, the higher prevalence is not only due to the lack of awareness and management of the disease but also the clinical heterogeneity where different subclasses have familial form and sporadic occurrence, which affect people of all ages. Our genetic screening of glaucoma, which included primary congenital glaucoma (PCG), primary open angle glaucoma (POAG), primary angle closure glaucoma (PACG), and pseudoexfoliation glaucoma (PEXG), involved replication studies, which revealed glaucoma-associated single nucleotide polymorphisms (SNPs) in the Pakistani population, exome sequencing resulted in novel gene identification. While global screening of glaucoma including the Pakistani cohort, led to the discovery of glaucoma-associated novel loci. All the studies performed until now have indicated genetics as one of the major components involved in familial and sporadic glaucoma onset in Pakistan.

18.2 Global Perspective

Glaucoma displays variable occurrence rate among different populations worldwide, similarly its genetic etiology exhibits heterogeneity and therefore remains largely unknown not only globally but also in different populations including Pakistan. Glaucoma is estimated to affect worldwide 79.6 million by 2020 [6] and 111.8 million by 2040 [7], three-fourth of whom will suffer from POAG [6]. Females are more prone to develop the disease, encompassing 70% of PACG patients, 55% of POAG, and 59% of other types of glaucoma. The ethnicity differences reveal that Africans have the highest occurrence rate, where POAG is observed to be more common, followed by Asians, where the Chinese population has a higher incidence of PACG patients, while normal tension glaucoma (NTG) subtype is more common among the Japanese [8].

Previously familial studies resulted in identification of 18 glaucoma loci (GLC1A to GLC1N,

GLC3A to GLC3D). The mutated genes included optineurin (*OPTN*), myocillin (*MYOC*), neurotrophin 4 (*NTF4*), and WD repeat domain 36 (*WDR36*) [9, 10]. Among the subclinical classes, PCG is more common in infants, usually occurring in sporadic manner in outbred populations, however, it is inherited recessively in inbred population [11, 12]. To date, four loci (GLC3A, GLC3B, GLC3C, and GLC3D) have been linked to PCG, with two identified genes GLC3A (*CYP1B1*) and GLC3D (Latent transforming growth factor beta binding protein; *LTBP2*) [13, 14]. Among the reported genes, cytochrome P450 (*CYP1B1*) has been found to be associated with PCG in different populations globally [9]. Through GWAS and case-control association studies in different cohorts, around 20 genes have been reported for POAG [9], while 9 genes have been found to be associated with PACG [15, 16]. More than 70 point mutations in *MYOC* have been found to be associated with POAG (predominantly 3–5% being associated with juvenile open angle glaucoma (JOAG)) [17] worldwide. Despite the identification of a number of genes, the complete genetic etiology of glaucoma remains undefined.

18.3 Epidemiology

A comprehensive report on glaucoma prevalence in Pakistan is still lacking, though small hospital-based studies have been conducted they do not provide a complete epidemiological overview of the Pakistani glaucoma patients. Therefore, there remains a gap in the determination of epidemiological basis of glaucoma of the Pakistani population. World health organization (WHO) national survey of blindness conducted between 1987 and 1990 showed that the prevalence of blindness varied from 2.0 to 4.3% in the Pakistani population, where glaucoma was found to be responsible for 3.9% blindness cases [18], which rose to 7.1% by the year 2007 [19]. However, another study has shown that 1.8 million people in Pakistan are affected by glaucoma among them one million are legally blind. Although it has a high prevalence, glaucoma is the major cause of treatable blindness in Pakistan second to cataract [19].

In Pakistan, POAG is the most frequent glaucoma subtype [20], especially in adults who are 70 years and older [19]. Moreover, PACG is more common among females and POAG among the males [21]. Congenital glaucoma accounts for up to 18% of childhood blindness and is believed to occur in 1 among 2000 births in the Middle East and 1 in 10,000 births in Western countries [22]. The higher occurrence of glaucoma in the Middle East is attributed to higher percentage of consanguineous marriages, which is even higher in South Asian countries including Pakistan [23].

18.4 Etiology

The etiology of glaucoma is complex with involvement of a combination of factors resulting in a similar pathological outcomes. The major risk factor of high prevalence of isolated primary glaucoma in Pakistan is old age [24]. Besides that, based on the recent genetic studies, the role of genetic susceptibility is becoming apparent. The major cause of familial glaucoma in Pakistan is consanguinity that results in many diseased recessive families, though dominant glaucoma families have also been identified worldwide [25], we also observed few dominant conditions in our studied cohort. Pakistan has one of the highest rates of consanguinity, because of which the ratio of PCG is higher followed by JOAG. Due to excessive inbreeding, clinical extremes have been observed in Pakistani patients. Among the environmental factors, exposure to sun also plays a role in disease manifestation as the people in the rural areas are usually farmers and are excessively exposed to sunlight. The major reason of occurrence of secondary form of glaucoma in Pakistan is use of steroid, for keratoconjuctivites, and allergies as well as self-medication for eye problems. The second most common cause of secondary glaucoma found in the Pakistani population is bilateral penetrating keratoplasty [26]. Besides, diabetes, cataract, and other diseases may also lead to secondary form of neovascular

glaucoma [27]. Due to a lack of awareness, delay in diagnosis, and treatment facilities in Pakistan, patients lose eyesight thus worsening their condition [28]. In addition, there are a number of systemic disorders called as glaucoma syndromes, which involve ocular abnormalities as a secondary feature [29]. These include Marfan's syndrome, Alfred Reiger's syndrome, Nail-patella syndrome, and Pigment Dispersion syndrome.

Though glaucoma induced blindness is preventable, numerous studies have demonstrated that access to glaucoma care facilities and noncompliance to therapy are still the major issues to be addressed in Pakistan. Several interrelated factors may contribute to noncompliance, including illiteracy in patients, self-medication, and poor socioeconomic status, which may be one of the reasons behind discontinuation of medicines resulting in progression of glaucoma to end stage. All these factors must be investigated in a glaucoma patient's noncompliance to medical treatment [30].

18.5 Experimental

In order to study the glaucoma genetic spectrum in the Pakistani population, we genetically analyzed familial and sporadic glaucoma, including the major clinical subclasses PCG, POAG, PACG, and PEXG. The genetic screening was started in 2008 and is still ongoing. In addition to that, all the glaucoma genetic findings by other groups working on Pakistani patients were also collated with our data in the current study to better understand the genetic etiology of the Pakistani population.

18.5.1 Identification of Novel Genes, Loci, and Novel Mutations in Known Genes

18.5.1.1 Homozygosity Mapping and Exome Sequencing

Glaucoma families were screened by exclusion mapping using Sanger sequencing, in these families, glaucoma was the primary cause of vision loss and also a secondary clinical feature in syndromic families. Using this technique few novel mutations in known genes were identified. The unsolved families were further analyzed by whole exome sequencing and homozygosity mapping after microarray analysis of selected family members, this resulted in identification of a few novel genes/loci (Table 18.1).

18.5.1.2 Genome Wide Association Studies

For the association studies, multiple techniques were used such as genome wide association studies (GWAS) [31, 32] that led to identification of novel loci (Table 18.2). Replication studies of selected SNPs based on their previous association with various populations were done through TaqMan/KASPAR assays, this highlighted the previously identified glaucoma associated SNPs role in the Pakistani population (Table 18.2).

18.6 Genetic Aspects

18.6.1 Familial Glaucoma Genetics in Pakistan

18.6.1.1 CYP1B1 Associated Glaucoma Families

Due to consanguinity in Pakistan, there is a frequent transmission of mutations through the generations resulting in a higher prevalence of genetic diseases. Many genes are expected to be involved in the progression of familial glaucoma in the Pakistani population, therefore the exact genetic cause remains undefined [33]. The GLC3A locus on chromosome 2 has been reported to be the most significant contributor to recessive PCG in the Pakistani population [34, 35]. *CYP1B1* is the gene that resides in the GLC3A locus and is one of the major causes of glaucoma [(PCG (34.6%) and POAG (3.3%)] in the Pakistani population (Fig. 18.1). The founder mutation p.Arg390His in *CYP1B1*, is the most frequent *CYP1B1* mutation not only in the Pakistani population (45%; Fig. 18.1) but

Table 18.1 Genes and their identified mutations causative of familial glaucoma in the Pakistani population

Gene (MIM ID)	Mutation (protein variation)	Phenotype	Chromosomal location	References
CYP1B1 (MIM: 601771)	p.Leu177Arg	PCG	2p22.2	[37]
	p.Leu487Pro	PCG		[37]
	p.Asp374Glu	PCG		[37]
	p.Arg390His[a]	PCG+POAG		[35, 38–42]
	p.Arg355*	PCG		[38, 40]
	p.Glu229Lys	POAG		[38–40]
	p.Ala288Pro	PCG		[38]
	p.Asp242Ala	PCG		[38]
	p.Arg290Profs*37	PCG		[38]
	p.Asp316Val	POAG		[38]
	p.Ala115Pro	PCG		[39]
	c.868_869insC	PCG		[39]
	p.Gly36Asp	PCG		[39]
	p.Gly67-Ala70del	PCG		[39]
	p.Trp434Arg	PCG		[35]
	p.Arg444Gln	PCG		[35]
	p.Tyr81Asn	PCG		[35]
	p.Arg368His	PCG		[35, 40, 41]
	p.Trp246Leufs81* + p.Glu299Lys	PCG		[35, 41]
	p.Pro442Glnfs15*	PCG		[35]
	p.Gln37*	PCG		[35]
	p.Arg469Trp	PCG		[35]
	p.Thr404Serfs30*	PCG		[35]
	c.1044-1G>C	PCG		[40]
	p.Gly61Asp			
	p.Pro437Leu	PCG		[42]
	p.Pro350Thr + p.Val364Met	PCG		[42]
	p.Leu13*	PCG		[42]
LTBP2 (MIM: 602091)	p.Arg299X p.Ala138Profs*278, p. Gln111X p.Glu415Argfs*596 p.Arg1645Glu p.Asp1345Glyfs*6	PCG	14q24.3	[44]
		PCG		
		PCG		
		PCG		
		PCG		[45]
		PCG		
MYOC (MIM: 601652)	p.Thr377Arg	JOAG	1q24.3	[47]
PXDN (MIM: 605158)	p.Gly1166Arg	PCG	2p25.3	[45]
PRPF8 (MIM: 607300)	p.Pro13Leu and p.Met25Thr	POAG	7q31.2	[46]
FOXC1 (MIM: 601090)	p.Ala31_Ala33del	PCG	6p25.3	[49]
PAX6 (MIM: 607108)	p.Tyr75*	Axenfeld-Rieger syndrome	11p13	[49]

(continued)

Table 18.1 (continued)

Gene (MIM ID)	Mutation (protein variation)	Phenotype	Chromosomal location	References
FBN1 (MIM: 154700)		Marfan's Syndrome	15q21.1	[50]
MYO18A (MIM: 610067)	p.Arg691Cys	JOAG	17q11.2	Ayub et al. [unpublished data]
ENOX1 (MIM: 610914)	p.Met57Ile		13q14.11	
COL9A2 (MIM: 120260)	p.Pro354Leu		1p34.2	
NCOA7 (MIM: 609752)	p.Val242Met	Late onset POAG	6q22.31-q22.32	Ayub et al. [unpublished data]
PHKG1 (MIM:172470)	p.Thr42Met	JOAG	7p11.2	Ayub et al. [unpublished data]
Novel Locus		PCG	14q24.2-24.3	[48]
Novel Locus		PCG	7q34	Ayub et al. [unpublished data]

'*' Stop codon, Under mutation section "+" indicates occurrence of two mutations in a single family, Under the phenotype section "+" indicates the coexistence of mentioned phenotypes in the family
[a]Founder mutation of Pakistani population

is also frequently reported in Saudi and South Korean populations [36]. The first report of the involvement of *CYP1B1* in glaucoma families of Pakistani origin was in 2008 with identification of three novel missense mutations (p.Leu177Arg, p.Leu487Pro, and p.Asp374Glu) in the gene [37]. Exclusion mapping that we performed in our cohort of 40 glaucoma families (12 PCG and 28 POAG) revealed one known and three novel homozygous mutations in *CYP1B1* in four PCG families (p.Arg355*, p.Ala288Pro, p.Asp242Ala, and p.Arg290Profs*37). The p.Arg390His is the most frequent mutation that we identified in our cohort [38]. In addition in a panel of POAG families, a novel heterozygous missense mutation (c.947A>T; p.Asp316Val) was identified along with a known mutation (p.Glu229Lys). The latter was also found in three other POAG families [38].

A study conducted by Sheikh et al. [39] on a panel of 20 PCG families that were screened by short tandem repeat (STR) markers spanning *CYP1B1,* revealed linkage of half of the panel (ten families) homozygously to *CYP1B1* region. Six mutations were identified in the *CYP1B1* linked families, with p.Arg390His being the most frequent mutation. Rauf et al. [35] identified two novel mutations p.W246Lfs81* and p.P442Qfs15* in *CYP1B1* in PCG families whereas nine recurrent mutations in their panel of 23 PCG families were identified where the founder p.Arg390His mutation was found to be segregating in 13/23 families. In another study by Afzal et al. [40] on a panel of 38 PCG families, ten families showed linkage to *CYP1B1* with the identification of one novel mutation (c.1044-1G>C) in the 3′ splice site in one family, while three other had recurrent mutations. Bashir et al. [41] recently identified a novel (c.736dupT, p.W246LfsX81*) and recurrent mutations in the *CYP1B1* in five out of six PCG families in their panel. In a recent study, direct sequencing of 11 PCG families for *CYP1B1* resulted in identification of mutation in seven families, this included a novel mutation p.P437L, compound heterozygous variants p.P350T and p.V364M as well as two known mutations p.R390H and p.P437L [42].

Table 18.2 Sporadic glaucoma associated novel genes and loci through genome wide association studies and replication studies

Gene	SNP ID	Glaucoma subtype	OR (95%CI) *p*-Value	References
CHAT (MIM:254210)	rs1258267	PACG	1.22 (1.58–3.98) 4.99×10^{-16}	[31]
POMP (MIM: 601952)	rs7329408	PEXG	1.13 (1.07–1.19) 1.61×10^{-5}	[32]
TMEM136 (MIM: 614465)	rs11827818	PEXG	1.15 (1.08–1.22) 4.35×10^{-6}	
AGPAT1 (MIM: 603099)	rs3130283	PEXG	1.24 (1.14–1.34) 2.27×10^{-7}	
RBMS3 (MIM: 605786)	rs12490863	PEXG	1.12 (1.05–1.20) 0.00053	
near *SEMA6A*	s10072088	PEXG	0.88(0.81–0.96) 0.0024	
ASB10 (MIM: 602432)	rs2253592	POAG	$P = 0.047$	[51]
TMCO1 (MIM: 213980)	rs4656461	POAG[a] PACG PEXG[a]	0.57(0.38–0.89) 0.003 0.52 (0.30–0.88) 0.009 0.54 (0.32–0.92) 0.01	[52]
ATOH7 (MIM: 221900)	rs1900004	PACG[a]	0.69(0.48–1.00) 0.03	[52]
CAV1 (MIM: 606721)	rs4236601	POAG	2.46 (1.01–6.24) 0.02	[52]
BIRC6 (MIM: 605638)	rs2754511	PEXG	0.42 (0.22–0.81) 0.05	[59]
XRCC1 (MIM: 617633)	rs25487	POAG	2.65(1.44–4.85), $p < 0.005$)	[53]
XPD (MIM: 278730)	rs13181	POAG	1.89 (1.23–2.9), $p = 0.005$)	[53]
LOXL1 (MIM: 177650)	rs1048661	PEXG	2.98 (1.94–4.57) 0.0001	[54]
	rs3825942	PEXG	6.83 (2.94–16.67) 0.00001	[54]
MTHFR (MIM: 181500)	rs1801133	PACG	1.09 (0.64–1.84) 0.001	[58]
NOS3 (MIM: 104300)	27bp intron 4 VNTR	POAG PACG PEXG	1.74 (1.10–2.75) 0.01 2.09 (1.23–3.55) 0.001 1.68 (1.01–2.7) 0.04	[55], Ayub et al. [unpublished data]
HSP70 (MIM: 140550)	rs1043618	POAG PACG PEXG	2.68 (1.79–4.01) 2.22 e^{-09} 1.91(1.18–3.10) 0.002 2.87 (1.75–4.71) 2.5 e^{-07}	[55], Ayub et al. [unpublished data]
COL11A1 (MIM: 228520)	rs3753841	PEXG	0.44 (0.19–1.0) 0.05	Ayub et al. [unpublished data]
GST (MIM: 138350)	M1	PEXG	20.77 (2.45–460.38) 0.001	[60]
	T1	PEXG	4.47 (1.96–10.29) 0.001	[60]

(continued)

Table 18.2 (continued)

Gene	SNP ID	Glaucoma subtype	OR (95%CI) *p*-Value	References
TNFα (MIM: 157300)	G-308A	PEXG[a]	0.24 (0.12–0.51) <0.001	[56]
MYOC (MIM: 137750)	rs74315341		197.01 $p = 0.04$	[33]
	rs879255525		199.25 $p = 0.016$	[33]
MMP1 (MIM: 226600)	rs1799750	POAG	2.14 (1.10–4.15) 0.001	[57]
MMP7 (MIM: 178990)	rs17576	PACG	1.34 (0.73–2.47) 0.35	[57]
CYP1B1 (MIM: 231300)	rs2567206	PEXG[a]	0.44 (0.25–0.77)/0.0002	Ayub et al. [unpublished data]

[a]Protective role

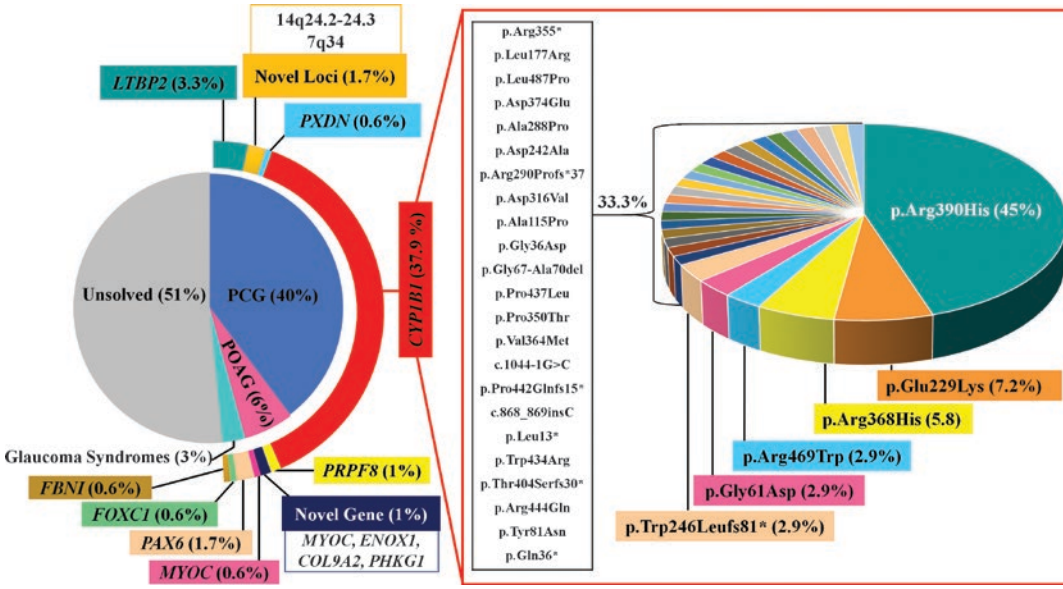

Fig. 18.1 Frequency of genes associated with PCG, POAG, and glaucoma syndromes in the Pakistani population in a cohort of 182 families. *CYP1B1* is the major cause of PCG and POAG. Frequency of the *CYP1B1* glaucoma causing mutations also represented, which accounts for 37.9% of families carrying *CYP1B1* mutations in the Pakistani Population (data compiled from studies [35, 37–42, 44–50])

Though involvement of *CYP1B1* is apparent in PCG in Pakistan, interestingly, our group identified homozygous *CYP1B1* mutation p.Arg390His in a large consanguineous family that had heterogeneous clinical presentation. The members were affected with POAG (both Juvenile and late onset) as well as PCG [38]. Similarly, a homozygous mutation c.182G>A, p.G61E in *CYP1B1* was also found to be responsible for both Juvenile onset POAG (27 years) as well as PCG in another consanguineous Pakistani family [43]. We also identified a novel heterozygous missense mutation (p.Asp316Val) in a late-onset POAG family thus extending the mutation spectrum of *CYP1B1* in Pakistani glaucoma families [38].

Data pooling from the studies done on glaucoma families of Pakistani origin revealed that since 2008 till date nearly 182 families have been genetically screened, where mutations in *CYP1B1* were found in 37.9% of the families (Fig. 18.1), most of these families were screened by direct *CYP1B1* sequencing, in the remaining families

(51%) there might be a deep intronic variant in *CYP1B1* causative of the disease or involvement of some other gene or some nongenic part. Therefore, there is a possibility of novel gene discovery in the unsolved PCG families.

18.6.2 Other Genes Involved in Familial Glaucoma

Second most frequently mutated gene (3.3%) in the PCG families was *LTBP2* (latent transforming growth factor beta binding protein, Fig. 18.1). To date, 6 mutations in *LTBP2* have been identified to cause PCG in Pakistan; Ali et al. [44] identified a homozygous nonsense mutation in exon 4 (c.895C >T; p.Arg299X), a homozygous single base pair deletion in exon 1 (c.412delG; p.Ala138Profs*278), a homozygous nonsense variant in exon 1 (c.331C>T; p.Gln111X) and a homozygous 14-base pair deletion in exon 6 (c.1243-1256 del; p.Glu415Argfs*596) [44]. In our cohort, a missense mutation (c.4934G>A; p.Arg1645Glu) and a novel frameshift mutation (c.4031_4032insA; p.Asp1345Glyfs*6) were also identified in *LTBP2* after whole exome sequencing of PCG Pakistani families [45]. Another family in our cohort was linked to *PXDN* with a novel missense mutation (c.3496G>A; p.Gly1166Arg) [45]. *PRPF8* that was previously identified to cause retinitis pigmentosa was found to be causative of POAG in Pakistani families, where two nonsynonymous variants p.Pro13Leu and p.Met25Thr were identified to be segregating with the POAG phenotype [46]. Another gene, *MYOC*, has also been reported to cause glaucoma in Pakistan with the identification of a heterozygous mutation (p.Thr377Arg) in a family with severe glaucoma phenotype [47].

The locus 14q24.2–24.3 was found segregating in two consanguineous Pakistani families in a study conducted in 2008 [48]. In another study [Ayub et al. unpublished data], homozygosity mapping revealed a novel locus 7q34 to be present homozygously in the affected members of a small PCG family. Though targeted exome sequencing was performed of the 2 MB locus, no plausible disease-associated gene was identified thus indicating the possible involvement of deep intronic mutation or nongenic region or some other gene outside of this region.

We also obtained interesting results with whole exome sequencing of POAG families [Ayub et al. unpublished]. In a large consanguineous dominant POAG family, we identified three variants in three novel genes (*MYO18A*: c.2071G>A; p.Arg691Cys, *ENOX1*: c.171C>T; p.Met57Ile, *COL9A2*: c.1061C>T; p.Pro354Leu) segregating with the disease. The presence of all the variants resulted in the early onset of the disease (discussed in clinical part). Whereas another variant (*NCOA7*: c.724C>T; p.Val242Met) segregated in a different loop of the same family, in this branch the three variants did not exist together and the affected persons had late onset of the disease. In another consanguineous Pakistani family with juvenile-onset POAG, inherited dominantly, whole exome sequencing identified a variant (c.125C>T p.Thr42Met) in the *PHKG1* to be segregating heterozygously [Ayub et al. unpublished data].

18.6.3 Genetics of Glaucoma Syndromes

Genes have also been identified in various glaucoma syndromes in the Pakistani population. We conducted a study of 14 Pakistani families presented with Axenfeld Rieger Syndrome along with presentation of Glaucoma as one of the complications of the syndrome. A novel homozygous deletion (c.92_100del; p.Ala31_Ala33del) was identified in the *FOXC1* segregating in a family with congenital glaucoma presentation [49]. Another family carried a de novo mutation c.225C>A; p.Tyr75* in *PAX6* causative of glaucoma in syndromic form. The mutation was not present among the parents of the proband [49]. We also identified a novel heterozygous missense mutation c.2368T>A; p. Cys790Ser in *FBN1* in a Marfan's syndrome family of Pakistani origin [50].

The familial form of PACG is rare, though in our panel of glaucoma families, we identified two small families that were screened for plausible

genes, but no known or novel variants were identified in these families [Ayub et al. unpublished data], thus indicating involvement of novel genes in PACG families.

18.6.4 Genetics of Sporadic Glaucoma

In order to determine a comprehensive genetic overview of glaucoma in the Pakistani population, our group conducted a number of case-control association studies that resulted in identification of pathogenic as well as protective SNPs in the Pakistani population (Fig. 18.2). GWAS conducted for the identification of genetic risk factors of sporadic glaucoma subtype PACG in worldwide and Pakistani PACG patients resulted in the identification of five novel loci, *EPDR1* (rs3816415) *CHAT* (rs1258267), *GLIS3* (rs736893), *FERMT2* (rs7494379), and *DPM2–FAM102A* (rs3739821) to be causative of PACG globally, however, in the Pakistani population only *CHAT* (rs1258267) was significantly associated [31]. While another GWAS of sporadic PEXG [32], revealed novel loci; 13q12 (*POMP*), 11q23.3 (*TMEM136*), 6p21 (*AGPAT1*), 3p24 (*RBMS3*), and 5q23 (near *SEMA6A*) along with a rare protective allele at *LOXL1* (p.Phe407) [32]. Replication association studies revealed variants in *ASB10* to be associated with POAG [51], *TMCO1* (rs4656461) with POAG, PACG as well as PEXG, *ATOH7* (rs1900004) with PACG, and *CAV1* (rs4236601) with POAG [52]. Polymorphisms rs25487 in *XRCC1* and rs13181 in *XPD* were found to increase the risk of POAG in males [53], while *LOXL1* SNPs rs1048661 and rs3825942 did not show any gender bias and were found to be only associated with PEXG [54], while a 27-bp intron 4 VNTR polymorphism in *NOS3* and *HSP70* rs1043618 polymorphism were found to be associated with POAG and PACG [55], as well as with PEXG [Ayub et al. unpublished]. SNP rs3753841 in *COL11A1* was also found to be significantly associated with PACG [Ayub et al. unpublished data]. *TNFα* polymorphism G-308A was associated with PEXG [56], while rs74315341 and the novel SNP rs879255525 in *MYOC* increase the risk of POAG in the Pakistani population [33]. *MMP1* polymorphism rs1799750 was found associated with POAG, *MMP9* polymor-

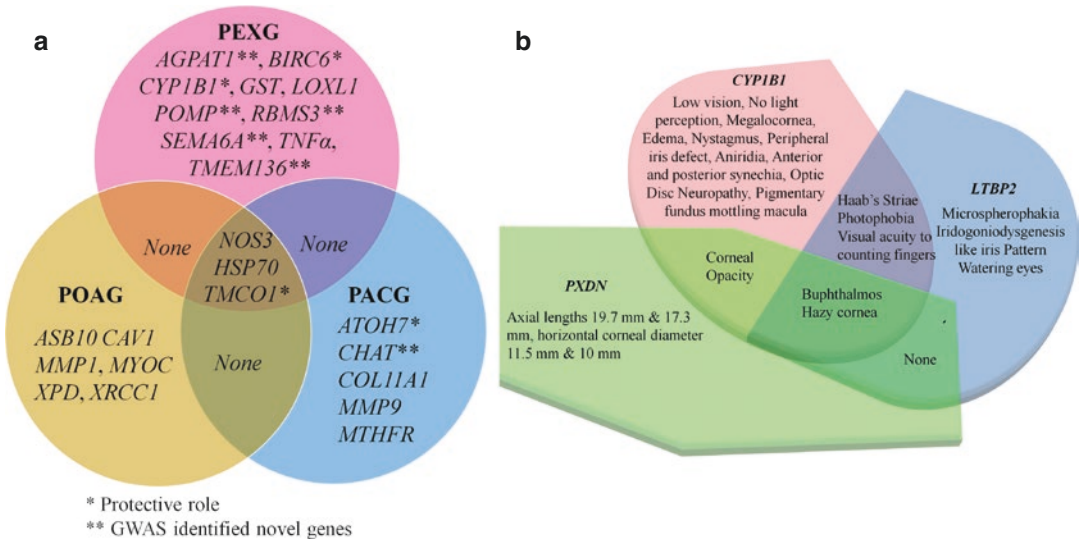

Fig. 18.2 (**a**) Genotype and phenotype correlations among the PCG families linked to the three genes *CYP1B1, PXDN, LTBP2* in the Pakistani population. (**b**) Genes, associated with three subtypes of Glaucoma: PEXG, POAG, and PACG, in the Pakistani Population

phism rs17576 with PACG [57]. *MTHFR* C677T polymorphism was found to be associated with PCAG but not POAG [58]. Whereas the *BIRC6* polymorphism rs2754511 was found to play a protective role in PEXG [59], and *CYP1B1* (*P450*) polymorphism rs2567206 also played a protective role in PEXG [Ayub et al. unpublished]. Moreover, *GSTT1* and *GSTM1* null genotypes were also found to be associated with PEXG in the Pakistani cohort [60].

There were few SNPs such as rs11720822 in *PDIA5* [59], rs11258194 in *OPTN, P21* polymorphism rs1801270, *P450* c.-2805T>C (POAG and PACG only), *CYP1B1* polymorphism rs2567206, rs1015213 in *PCMTD1,* rs11024102 polymorphism in *PLEKHA* that were not found to be associated with glaucoma in Pakistani population, despite their disease association in other ethnicities worldwide [Ayub et al. unpublished].

Polymorphic genetic variations in different genes that were observed to play a genetic role in sporadic glaucoma in the Pakistani population are listed in Table 18.2.

18.7 Pathology and Clinical Features

Different forms of glaucoma share some common clinical features that include changes in cup-to-disc ratio (CDR), thinning of retinal nerve fiber, which happens due to the loss of optic nerve and RGCs. The visual field loss initiates in the periphery until only the central vision is left [27]. Various forms of glaucoma exist in Pakistan, the most common clinical presentation among the children is PCG while POAG is common in adults. Based on the genetic findings there exists a genotype–phenotype correlation in different forms of glaucoma in Pakistan.

18.7.1 Genotype–Phenotype Correlation

Like genetic heterogeneity, the clinical presentation of glaucoma was also observed to be heterogeneous in the Pakistani glaucoma patients (Fig. 18.2b). In familial glaucoma subjects, Waryah et al. [42] observed varying degrees of onset and severity of disease in their studied families, where most of the families had early disease onset. In our cohort, a large family of POAG with a dominant mode of inheritance, had differences in the age of onset of the disease among various affected individuals among the sub-branches of the family. One sub-branch displayed early onset of the disease with an average age of 25 years while the other sub-branch had late disease onset with mean age of 50 years. The patients had severe phenotype with raised IOP, pain in eyes and forehead region and tunnel vision. The symptoms were severe in one of the patients with early onset of the disease; the affected girl had onset in the first decade of life with severe clinical symptoms and rapid loss of vision [Ayub et al. unpublished]. Another dominant late-onset POAG family had blurring of vision and persistent headache at the beginning of the disease. They had high intraocular pressure (IOP), retinal nerve fiber layer (RNFL) thinning, and appearance of clinical symptoms in fourth to fifth decade of life. However, a child (7 years at the time of sampling) in the family had loss of vision with excessively raised IOP and deep cupping, with appearance of clinical symptoms in the first decade of life. The child was also observed to be homozygous for the identified segregating mutation in *PHKG* while other affected individuals in this family were heterozygous [Ayub et al. unpublished data]. The families that were linked to *CYP1B1* had varying clinical phenotypes, one of them with a mutation (p.Arg390His) in *CYP1B1* had family members affected with PCG, JOAG, and late-onset POAG as well. "The patient having PCG had megalocornea, hazy cornea, edema, raised IOP (32 mmHg) and nystagmus, whereas the patients with late-onset POAG in the same family had poor vision, a phthisical left eye, nystagmus and optic disc neuropathy with a pigmentary mottling of the fundus in the macula of her right eye. The individual with JOAG onset had phthisical left eye, a peripheral iris defect, aniridia and anterior and posterior synechia, and a high IOP

(44 mmHg) of the right eye, while another individual had megalocornea, nystagmus, raised IOP in both eyes (35 and 30 mmHg) and a CDR of 1.0 in both eyes" [38]. In the same study, 4 PCG families were linked to *CYP1B1* (Fig. 18.2b). The age of onset was before 3 years with very high IOP (>40 mmHg), bulging eyes (buphthalmus), with varying opacity, horizontal corneal diameters were 13 mm [38] (Fig. 18.2b). Clinical variability has been observed among patients with variants in *CYP1B1* in the Pakistani population [41] (Fig. 18.2b). A PCG family with two affected individuals who suffered from glaucoma in first year of life had raised IOP (>40 mmHg) and nystagmus bilaterally. The family was genetically analyzed by homozygosity mapping that resulted in identification of a novel locus [Ayub et al. 2019 unpublished]. Both individuals had bulging avascularized and opaque corneas with sensitivity to touch and Haab's striae (horizontal breaks in the Descemet's membrane). The disease was progressive that resulted in complete blindness while the unaffected siblings had no signs of glaucoma. In other populations worldwide, patients with *PXDN* mutations have been observed to display severe anterior segment dysgenesis and microphthalmia [61], however, in our Pakistani cohort we observed anterior segment dysgenesis, sclerocornea, microphthalmia, hypotonia, and developmental delays among the patients [38]. Moreover, overlapping clinical features were observed among the probands of the PCG families that were linked to different genes (Fig. 18.1). Familial glaucoma clinical presentation is therefore observed to be complex where single gene defect results in differential phenotype among the family members, which points to the involvement of genetic modifiers in glaucoma progression, therefore, there is a need to identify these modifier genes to add to the understanding of the genetic etiology and hence the molecular mechanisms of glaucoma.

Among the sporadic cases that we studied, most of the patients came to the clinics with compromised vision. In the studied sporadic cohort, we observed comorbidity of NTG and high tension glaucoma (HTG) among the POAG group. Among these patients, the CDR ranged from 0.4 to 1 with increased vision loss along with increasing CDR. The patients with late-onset POAG were mostly blind due to glaucoma when they first visited the clinics. The JOAG patients usually had a family history and raised IOP with disturbed CDR (>0.5). Among the PACG patients, the IOP was observed to be very high, i.e., above 40 mmHg with red and watery eyes. The PACG patients usually had severe loss of vision, which in some cases was accompanied by excessive optic nerve damage, whereas patients of PEXG had exfoliation deposits in the TM and other aqueous bathed surfaces and usually had late-onset open angle glaucoma with raised IOP (<30) and disturbed CDR (>0.5).

The sporadic Pakistani glaucoma patient's awareness about glaucoma onset, progression and its consequences were poor as compared to the patients with family history. The former came to the clinics usually with compromised vision and poor understanding of the fact that vision restoration is not possible for a glaucoma patient and thus it becomes a major cause of depression among glaucoma patients.

Better understanding of the pathology of glaucoma therefore can help in improvement in management and treatment of the disease. In Pakistan, treatment generally includes medication, targeting, and reducing IOP by topical medicine, laser or surgical procedure [62]. β-blockers (timolol and betaxolol) are most commonly used to lower the IOP, along with α2 adrenoreceptors, which lower the IOP by inhibiting the aqueous humor inflow [63]. In case of failure of medicines and laser treatment or in very severe disease condition, trabeculectomy is the procedure of choice to lower the IOP and trabeculectomy with 5-FU is an efficient surgical procedure for glaucoma treatment in practice in Pakistan [64]. However, despite multiple treatment methods it has been observed that a number of patients respond to the medication differently, being categorized as responders and nonresponders to treatment. Such observations, therefore, point toward the need of exploring pharmacogenetic aspect of glaucoma in the Pakistani patients.

18.8 Molecular Biology

18.8.1 Molecular Biology of Familial Glaucoma in Pakistan

The major gene contributing to PCG in the Pakistani population and other ethnicities worldwide is observed to be *CYP1B1*. However, in the Pakistani population, we also observed *CYP1B1* mutations in POAG families thus extending the disease spectrum of *CYP1B1* to glaucoma subtypes. The gene belongs to the family of cytochrome *P450* [65] and the protein is essential in the proper development and functioning of the iridocorneal angle of the eye [66] and maintenance of the trabecular meshwork (TM), which is the most significant tissue with respect to glaucoma [67]. The mutated *CYP1B1* is predicted to result in disruption of TM cell arrangement at early developmental stages that interrupts the aqueous humor outflow resulting in elevated IOP in PCG as well as POAG patients [67]. The involvement of *CYP1B1* mutations in a POAG family that we studied where patients displayed clinical variability (late-onset POAG, JOAG, and PCG), points toward the involvement of a modifier gene, or it might be due to the interaction of environmental factors (xenobiotics or mutagenic chemicals) [68].

Mutations in *LTBP2* and *PXDN* (also reported in the Cambodian population [69]) have been found to be causative of PCG in the Pakistani population. LTBP2 is expressed in the TM ciliary processes where it has a vital function in the production and maintenance of aqueous humor, it is also involved in tissue repair and cell to cell adhesion [70]. The involvement of *LTBP2* in the developing elastic tissues is postulated as the molecular mechanism causative of PCG [71]. *LTBP2* also interacts with *FBN1* (which causes Marfan's syndrome) [72, 73] to maintain integrity of the extracellular matrix (ECM) [74]. Though the pathogenic mechanism for both LTBP2 and PXDN is unclear in glaucoma but they are both predicted to be linked to each other through COL4A2 and the assembly of ECM in the TM which is important in maintenance of IOP. *PRPF8*, is another gene, mutations in which have been found to be causative of POAG [46], this gene was previously reported as RP causing gene in retinal dystrophy families [75], this is a novel genetic association for POAG manifestation. *PRPF8* interacts at its C and N terminals with the interacting partners (U2-dependent spliceosome complex composed of four snRNPs; U1, U2, U4/U6, and U5), which are important for splicing. Previously C terminus mutations in the gene were identified to be causative of RP [75] but in our cohort we found mutations in the N terminus to be causative of POAG [46], thus mutations on both termini are predicted to disrupt the normal protein function and disturb the interaction with other proteins, however, how the N and C terminus mutations result in different phenotype needs further investigation.

Despite the fact that a number of genes have been identified in Pakistani glaucoma families, the unsolved families (51%; Fig. 18.1) indicate the existence of un-identified gene(s) and pathways causative of glaucoma.

18.8.2 Molecular Biology of Sporadic Glaucoma in Pakistan

The two major primary forms of glaucoma, PACG and POAG have been extensively studied worldwide as well as in the Pakistani population, however, we extended our sporadic glaucoma cohort to a secondary form of glaucoma, i.e., PEXG. We reported our first association study on *MTHFR* in 2008 [58], with PACG and not with POAG, which was also the first genetic association report of sporadic glaucoma phenotype from Pakistan. The difference in the association of *MTHFR* for different glaucoma subclasses led us to explore further, the other genetic variants involved in various molecular pathways that have reported association in other populations worldwide. We studied various SNPs in those genes that had shown association in ER stress and apoptosis (*P53*, *P21*, *P450*, *PDIA5*, *BIRC6*, *OPTN*), genes expressed in Ciliary body, TM (*MYOC*, *ASB10*), genes involved in overcom-

ing oxidative stress (*eNOS*, *HSP70*, *GSTs*). Cell junction maintenance (*PLEK1*), collagen growth and repair (*COLL11A1*, *MMPs*), DNA repair pathway genes (*XRCC*, *XPD*), and inflammation (*PCMTD1*, *TNFα*).

These association studies helped us in the identification of population-specific SNPs. A global screening of PACG samples identified novel genes and pathways [31] in which five novel loci were found to be associated with PACG (Table 18.2). When population-based data were analyzed, only one SNP (*CHAT*; rs1258267) was found to be associated with PACG in the Pakistani population [31]. Moreover, when the global PEXG cohorts were screened, it also resulted in novel loci identification in the Pakistani population as well [32]. Thus, these studies helped in highlighting the pathways such as oxidative stress and inflammatory pathway that are associated with the disease in the Pakistani population. Further replication studies should be done in Pakistani as well as in other populations for the identification of population-specific genetic risk variants.

18.9 Summary

Pakistan, with one of the highest ratios of consanguinity, has a large number of genetic disease families including glaucoma. There is clinical as well as genetic variability among the patients of glaucoma in the Pakistani population. In certain cases, a single gene mutation might cause varying effects in the individuals, suggestive of the presence of modifiers that require further comprehensive studies to understand the complete mechanisms of glaucoma. Moreover, glaucoma awareness is the need of the hour and all modes of communication should be used to sensitize the public at large. The patients are not familiar with the genetic basis and the familial nature of the disease; therefore it has become a serious blindness-related issue in Pakistan despite advances in diagnostic, medical, and surgical treatment options.

Moreover, looking at the mutation spectrum of *CYP1B1* and the identification of novel mutations in the gene in Pakistani population, this gene should be prescreened for mutations in all new families of not only PCG but also POAG. Several novel mutations in novel and known genes identified in our cohort could not be functionally validated in the genetic studies conducted in Pakistan. Therefore, molecular characterization of glaucoma is essential, which can only be done through high throughput techniques. As glaucoma is a complex disorder therefore the best technique for its molecular characterization is whole genome sequencing, which will be helpful in understanding its genetic etiology. Functional studies including proteomic approaches and animal models would further be required for the validation of the genetic data, followed by further replication studies in different populations. Only then it would be possible to unravel the causative agents of these complex disorders and their underlying molecular mechanisms that will further help in developing better therapeutic interventions.

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Contributions of Promoter Variants to Complex Eye Diseases

19

Tsz Kin Ng and Chi Pui Pang

Abstract

Common eye diseases, including myopia, cataract, glaucoma, and age-related macular degeneration, are the leading cause of blindness and visual impairment, affecting billions of people worldwide. Unlike monogenic diseases, the inheritance of common eye diseases is complex, interplaying with genetics and environmental factors. Genome-wide association studies (GWAS) have identified hundreds of associated genes for common eye diseases; yet, the biological correlation of these disease-associated genes with the pathogenesis of the common eye diseases remains elusive. Apart from the involvement of multiple genes, the epigenetic regulation by environmental factors, including cigarette smoking and sunlight exposure, also determines the occurrence and etiology of the complex diseases. A gene promoter is composed of multiple transcription factor binding sites, which time-dependently regulates the spatial expression of a gene. Genetic variants in the promoter region, creating or disrupting the transcription factor binding sites, could impair the expression of the disease-associated genes and contribute to the pathogenesis of the common eye diseases. In this chapter, the association of the gene variants in the promoter region with the common eye diseases was summarized, with the focus on myopia, cataract, glaucoma, and age-related macular generation. In addition, the contribution of the promoter variants to the pathogenesis of these complex common eye diseases would also be discussed.

Keywords

Promoter · Genetic variants · Myopia Glaucoma · Age-related macular degeneration · Cataract

T. K. Ng (✉)
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong, Shantou, Guangdong, China

Shantou University Medical College, Shantou, Guangdong, China

Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, China

C. P. Pang
Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong, Shantou, Guangdong, China

Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, China

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19.1 Introduction

Myopia, cataract, glaucoma, and age-related macular degeneration (AMD) are the most common ocular disorders, affecting almost all human beings in the world during their lifetime. No matter what the disease onsets are, the influence of environmental factors, such as sunlight exposure, cigarette smoking and food intake, complicate the development and progression of these common eye diseases [1]. Besides, multiple etiology, clinical heterogeneity, overlap of phenotypic features as well as limited large pedigree also hinder the disease gene discovery for these complex eye diseases. Nevertheless, the major breakthrough in complex eye disease genetics begins with the International HapMap Project and the application of genome-wide association studies (GWAS) on AMD [2]. Since 2005, more than 300 genes were identified as the disease susceptible genes for different eye diseases. Unlike monogenic diseases, disease penetration is hard to be explained by a single associated variant of the disease susceptible gene. Besides, because of the strong linkage disequilibrium, the causal variant cannot be identified only by the statistical methods [3]. Furthermore, considering the late disease onset and interaction with environmental factors, the variants in the exons are less likely to be the causal variants for complex eye diseases.

The precise regulation of gene transcription and translation is the key to the central dogma of molecular biology. This relies on the cis-regulatory DNA elements as well as the epigenetic regulation to control the gene expression. Gene promoters with the enhancers and repressors are composed of multiple transcription factor binding sites, which time-dependently regulate the spatial expression of the genes. Genetic variations in the cis-regulatory elements would create or abolish the transcription factor binding sites, which would influence the transcription of the genes. Cumulative misexpression of the disease susceptible gene could lead to a disease phenotype when age advances. This also explains the small odds ratio (OR) of most GWAS-identified variants for the complex eye diseases. In this chapter, the genetic association of the variants located in the promoter region was summarized, with the focus on the common eye diseases, including myopia, age-related macular generation, glaucoma, and cataract. In addition, the contribution of the promoter variants to the pathogenesis of these complex common eye diseases would also be discussed.

19.2 Myopia

Myopia, one of the most common refractive disorders worldwide, is an epidemic public health issue, especially in Asia. High prevalence (80–90% in young adults; [4]) and fast progression of myopia [5] in East and Southeast Asian countries lead to the national defense and occupational problems as well as the economic burden to the society. Individuals with high myopia, defined as spherical equivalence below –6.0 diopter (D), are more prone to develop serious ocular complications, including macular hole, retinal detachment, glaucoma, premature cataract, and choroidal neovascularization [6], which could lead to irreversible visual impairment or even blindness.

The etiology of myopia is complex. Environmental factors and inheritance have been implicated in the development of myopia. Environmental factors, such as near work, outdoor activities, and sunlight exposure, could be attributed to the development of myopia [7], whereas high heritability of myopia has been observed from the twin and familial studies [8, 9]. Currently, more than 20 *MYP* loci have been mapped for myopia by the family linkage analysis [10]. Moreover, a recent GWAS with 255,925 study subjects identified 161 genetic variants significantly associated with refractive error [11]. These refractive error-associated genes cover the light-dependent signaling cascade from cornea to sclera, including rod-and-cone bipolar synaptic neurotransmission, anterior segment morphology, and angiogenesis. However, the functional consequences of these gene variants to the development of myopia still remain unknown. Besides, most of the associated variants are located in intergenic region, indicating the possible role of transcriptional regulation. In this section, the association of promoter variants in multiple genes with myopia was summarized and discussed.

19.2.1 Paired Box 6 Gene

Paired box 6 (*PAX6*) gene mutations were identified for the development of aniridia [12]; yet, the association of *PAX6* gene with myopia was initially discovered by a genome-wide scan of 506 twin pairs with the heritability of 0.89 in the British population [13]. Significant linkage with a maximum LOD score of 6.1 was identified on chromosome 11p13. Tag SNP analysis demonstrated five variants of *PAX6* gene explaining 0.999 of the haplotype diversity. However, in our Hong Kong Chinese cohort, no sequence alterations in the coding or splicing regions showed an association with high myopia [14]. Besides, tag SNP analysis indicated that there was no significant association of *PAX6* variants (rs2071754, rs3026354, rs3026390, rs628224, rs644242, and rs662702) with mild (–1.0 to –3.0 D), moderate (–3.0 to –6.0 D), and high myopia [15]. These *PAX6* variants were also not correlated with the axial length. In contrast, 2 *PAX6* intron variants (rs2071754 and rs644242) were found to be associated with extreme myopia (< –10.0 D) with odds ratio (OR) of 1.33. Moreover, the *PAX6* rs644242 variant could be associated with high myopia (OR = 0.87; dominant model) as well as extreme myopia (OR = 0.79; dominant model) as suggested by a meta-analysis of 6888 study subjects with Asian ancestry [16].

Although *PAX6* coding variants are not associated with myopia, there could be possibility of genetic variation in the upstream promoter or regulator. Our group identified two highly polymorphic dinucleotide repeats, AC_m and AG_n, in the P1 promoter region of the *PAX6* gene significantly associated with high myopia [14]. Higher numbers of both AC_m and AG_n repeats were observed in high myopia patients with an OR of 1.33. Our luciferase-reporter analysis further demonstrated elevated transcription activity with increasing individual AC_m and AG_n and combined AC_mAG_n repeat lengths, suggesting that higher expression of *PAX6* gene could be related to the development of high myopia.

Apart from the promoter variants, the microRNA binding site could also be involved in the regulation of *PAX6* gene expression. MicroRNA-328 binds to the wild-type C-allele, but not the T-allele of rs644242 variant [17]. Increased microRNA-328 expression suppresses *PAX6* expression and downregulation of *PAX6* reduces scleral cell proliferation. Collectively, promoter and microRNA regulations suggest that increased PAX6 expression is associated with myopia and its pathological changes; therefore, *PAX6* should play a role in myopia development.

19.2.2 Lumican Gene

The correlation of lumican (*LUM*) gene (chromosome 12q21.33) with myopia can be observed from the double knockout mice of lumican and fibromodulin ($Lum^{-/-}/Fmod^{-/-}$), which thinner sclera and increase in axial length were observed in $Lum^{-/-}/Fmod^{-/-}$ mice [18]. Similarly, knockdown of lumican gene (*lum*) in zebrafish by antisense morpholinos resulted in scleral thinning and increased size of scleral coats due to the disruption of the collagen fibril arrangement in the sclera [19]. However, *LUM* is not the candidate gene in the *MYP3* locus for high myopia [20]. On the contrary, a LUM promoter variant rs3759223 was first suggested to be associated with extreme myopia in the Taiwan population with a *p*-value of 2.83×10^{-4} [21]. A meta-analysis with 1545 Chinese subjects from five studies indicated that the C-allele of *LUM* rs3759223 variant is protective against high myopia with an OR of 0.53 [22]. Yet, the *LUM* rs3759223 variant is not associated with high myopia in the Korean population [23]. Another meta-analysis with 2297 subjects from six studies confirmed no association of *LUM* rs3759223 variant with high myopia in all genetic models [24].

In addition to the rs3759223 variant, another *LUM* promoter variant rs3759222 is also not significantly associated with high myopia in the Korean population [23]. In contrast, the haplotypes of *LUM* variants c.601, c.-59, c.-628, and c.-1554 are significantly associated with high myopia in the Taiwan population with an OR of 4.71 [25]. Apart from the promoter variants, a

3′-UTR variant (c.1567:C>T) showed a significant association with high myopia in the Taiwan population [26]. The T-allele of *LUM* c.1567 variant exhibits a lower reporter gene activity compared to the C-allele.

Collectively, although there is controversy in the association of *LUM* promoter variants with myopia, population-specific association could exist for different *LUM* promoter variants.

19.2.3 Extracellular Matrix-Related Genes

Laminin-α1 (*LAMA1*) gene on chromosome 18p11.31 is a candidate gene in the *MYP2* locus for high myopia. However, none of the variants across the *LAMA1* gene, including 2 promoter variants (rs334384 and rs334420), are associated with extreme myopia in the Japanese population [27]. Another *LAMA1* promoter variant rs2089760 has been shown to be associated with high myopia in the Chinese population with an OR of 1.38 [28]. This *LAMA1* promoter variant is located at the transcription factor binding site, which the A-allele of rs2089760 variant, compared to the wild-type G-allele, reduces transcription factor binding ability and transcriptional initiation activity, and negatively regulates the expression of *LAMA1* gene [29]. This indicated that reduced expression by *LAMA1* rs2089760 variant could be involved in the development of pathological myopia.

Although the expression of matrix metalloproteinase-2 (MMP-2), but not MMP-3, was found to be elevated in human aqueous humor of the myopic eyes [30], no significant association was detected for the promoter variants of MMP-1 (c.-1607), MMP-2 (c.-1306:C>T and c.-735C>T), and MMP-3 (c.-1612) with high myopia in the Japanese population [31]. The association of MMPs variants requires further confirmation in different populations.

No association of collagen type I alpha 1 (*COL1A1*) variant was identified with myopia in the Caucasian population [32]. Similarly, there is also no association detected for the *COL1A1* intron variant rs2075555 with high myopia [33]; yet, a meta-analysis of 1620 Asian subjects showed a significant association of *COL1A1* promoter variant rs2269336 with high myopia [34]. Moreover, increased methylation at the 6 cytosine-phosphate-guanine (CpG) sites in the promoter and exon 1 region of *Col1a1* gene was reported in the monocular form deprivation-induced mice, accompanied with reduction of scleral *Col1a1* mRNA when compared to the normal control mice [35]. These indicate that the variation in COL1A1 expression, especially in sclera, could be involved in the development of myopia.

19.2.4 Other Genes

Transforming growth factor-β-induced factor (*TGIF*) was first reported to be associated with high myopia in our Hong Kong Chinese cohort [36]. However, the *TGIF* promoter variant rs4797112 is not associated with ocular biometric measures and myopia in the Australian Caucasian cohort [37].

Myocillin (*MYOC*) is a disease-causing gene for primary open angle glaucoma [38]. Mild association was reported for the *MYOC* variants with high myopia in the Caucasian populations [39]. However, in our Hong Kong Chinese cohort, we did not find the association of a GT repeat from c.-339 to c.-314 in the *MYOC* promoter with myopia [40].

19.3 Age-Related Macular Degeneration

AMD is the leading cause of irreversible blindness and visual impairment in the elderly populations, which will affect 196 million people worldwide in 2020 [41]. According to the international classification and grading system of age-related maculopathy and AMD [42], early AMD is characterized by drusen as well as the hyperpigmentation and hypopigmentation of retinal pigment epithelium (RPE) in the macula. Advanced stage is divided into "non-neovascular" and "neovascular" AMD. Non-neovascular AMD is character-

ized by geographic atrophy of RPE with an oval hypopigmented spot in which large choroidal vessels are visible, whereas neovascular AMD is characterized by choroidal neovascularization (CNV), which could lead to the detachment of the neuroretina or RPE from Bruch's membrane by serous or hemorrhagic fluid. Current effective treatments are limited to the anti-vascular endothelial growth factor (VEGF) treatments against neovascular AMD, and there is still no proven therapy for non-neovascular AMD [43].

AMD is a late-onset and progressive disease. Clinical heterogeneity, overlap of phenotypic features, and gross interactions with environmental factors, such as smoking, body mass index, hypertension, and chronic inflammation, complicate the genetic investigations for AMD [44]. In spite of rare big pedigrees for family linkage analysis, a meta-analysis of genome scans has revealed chromosome 10q26 to be the strongest AMD susceptibility locus, whereas chromosomes 1q, 2p, 3p, and 16 are likely linked to AMD [45]. Yet, the major breakthrough in AMD genetics was achieved by GWAS since 2005. Currently, a large GWAS with 33,976 study subjects from the Caucasian populations identified 52 independently AMD-associated variants across 34 loci [46]. Moreover, the Genetics of AMD in Asians (GAMA) Consortium also identified three additional AMD loci in *C6orf223*, *SLC44A4*, and *FGD6* genes [47]. However, most of the associated variants are located in the intergenic regions or introns, suggesting the possibility of gene expression regulation by the cis-regulatory elements in these loci. In this section, the association of promoter variants in GWAS identified genes with AMD was summarized and discussed.

19.3.1 Complement Factor H Gene

Complement factor H (*CFH*) gene on chromosome 1q31 is the first AMD-associated gene identified by the GWAS analysis [48], which the p.Tyr402His variant (rs1061170) shows the strongest association with AMD in the Caucasian population (OR = 7.4). On the contrary, the p.Ile62Val variant (rs800292), instead of p.Tyr402His, is associated with neovascular AMD in our Hong Kong Chinese population [49]. In addition to the non-synonymous variants, we also identified 2 *CFH* promoter variants rs3753394 (c.-331T>C) and rs35836460 (c.-195T>C) significantly associated with AMD from the whole gene screening analysis [50]. The association of the *CFH* rs3753394 variant with AMD has been confirmed in the Sichuan Chinese [51] as well as the Northern Spanish populations [52]. The haplotype containing the C-allele of *CFH* rs3753394 variant confers a significant protection against AMD. Furthermore, a meta-analysis from 19 studies with 10,676 subjects identified a significant association of another *CFH* promoter variant (rs1410996; c.-543G>A) with AMD [53].

A 241-bp region from c.-416 to c.-175 of *CFH* promoter shows specific transcription factor binding activity with c-Jun and c-Fos in astrocytes [54], implying that *CFH* promoter variants rs3753394 and rs35836460 could influence the transcription and expression of *CFH* gene (Fig. 19.1). This could be further confirmed by another GWAS that *CFH* promoter variant rs3753394is significantly associated with the serum levels of C3 [55], which is negatively regulated by CFH protein. Collectively, *CFH* promoter variants should be involved in the regulation of *CFH* gene expression, which in turn regulates the activation of the alternative complement system by interacting with C3.

19.3.2 High Temperature Requirement Factor A1 Gene

The age-related maculopathy susceptibility protein 2 (*ARMS2*)/high temperature requirement factor A1 (*HTRA1*) locus on chromosome 10q26 is the second AMD-associated locus identified by GWAS from our Hong Kong neovascular AMD cohort [56]. Our previous meta-analysis confirmed the association of *HTRA1* rs11200638 variant (G>A) with AMD globally across different ethnic groups with an OR of 7.32 in the homozygous model [57]. The risk A-allele of *HTRA1* promoter variant rs11200638 variant

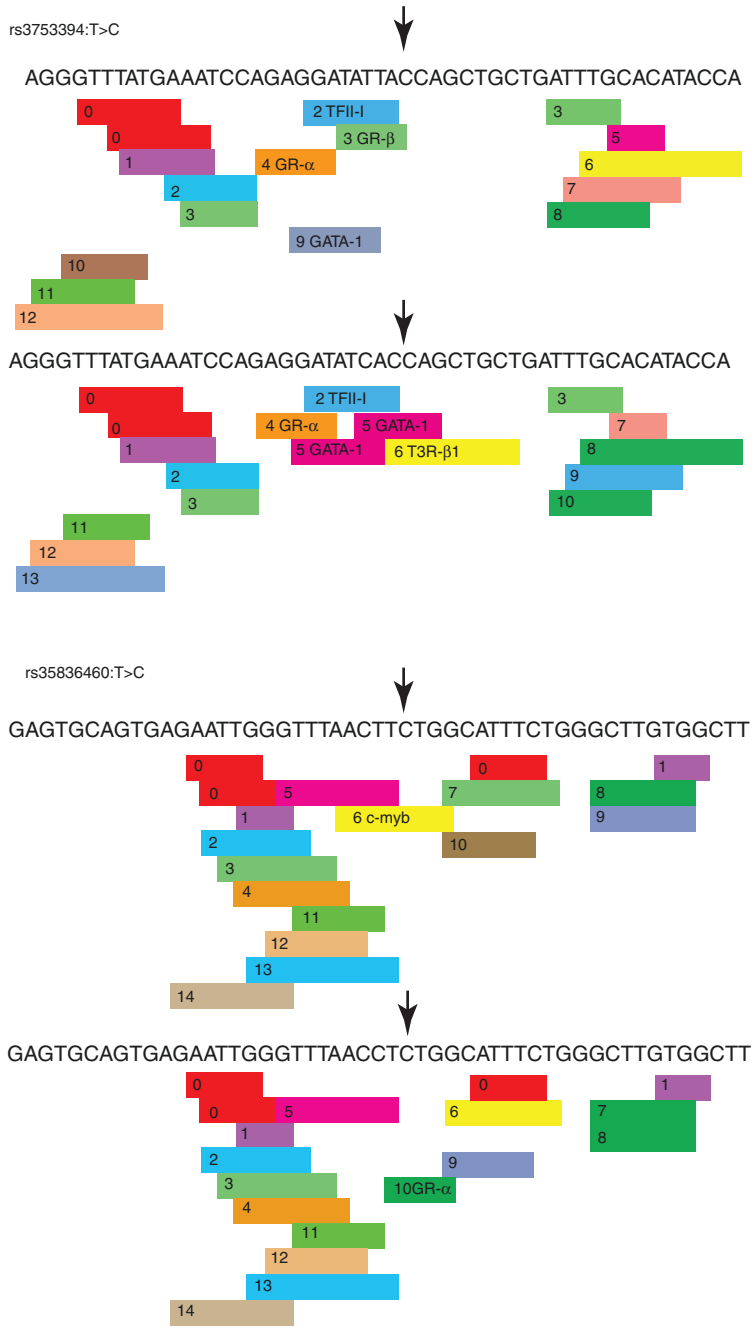

Fig. 19.1 Transcription factor binding site prediction on the AMD-associated complement factor H promoter variants. The GR-β site of the rs3753394 T-allele is predicted to be changed to one GATA-1 site and one T3R-β1 site at the rs3753394 C-allele. The c-Myb site of the rs35836460 T-allele is predicted to be changed to the GR-α site at the rs35836460 C-allele. The transcription factor binding sites were predicted by PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3/)

was demonstrated to increase the transcription activity of *HTRA1* promoter [56], and enhanced HTRA1 protein expression was detected in the retina from AMD patients [58]. Moreover, the *HTRA1* promoter variant rs11200638 increases the AMD susceptibility joint addictively with the *CFH* rs800292 variant (OR = 23.3) as well as smoking (OR = 15.71; [59]), but not with the cholesterol level [58]. In addition, the *HTRA1* promoter variant rs11200638 is associated with poorer visual acuity outcomes at 12 months, and the AMD patients with the homozygous AA genotype are more likely to lose more than 15 letters after 12 months [60]. The *HTRA1* promoter vari-

ant rs11200638 is also associated with a poorer response to the ranibizumab and bevacizumab anti-VEGF treatment for neovascular AMD.

Apart from the rs11200638 variant, we identified another common promoter variant rs2672598 (T>C) associated with neovascular AMD by whole gene sequencing analysis in our Hong Kong Chinese cohort [61]. The association of rs2672598 with neovascular AMD is independent of rs11200638; yet, the haplotype of the 2 *HTRA1* promoter variants rs11200638-rs2672598 (AA-CC) confers 43.11-folds of risk to neovascular AMD. Luciferase-report assay demonstrated that the C-allele of *HTRA1* rs2672598 variant shows higher luciferase expression than the wild-type T-allele (Fig. 19.2). In contrast, the luciferase expression levels are similar between the risk A-allele and the wild-type G-allele of *HTRA1* rs11200638 variant. Furthermore, the expression level of HTRA1 protein in vitreous humor with rs2672598 CC genotype was significantly higher than that with the wild-type TT genotype [61], whereas the rs11200638 genotypes are not correlated with the HTRA1 protein expression level in vitreous humor [62]. Furthermore, the C-allele of *HTRA1* rs2672598 variant was predicted to change the transcription factor binding sites of *HTRA1* promoter, whereas the A-allele of rs11200638 variant does not change the transcription factor binding sites. Therefore, we postulate that the *HTRA1* promoter variant rs2672598, instead of rs11200638, should be responsible for the elevated *HTRA1* transcriptional activity and HTRA1 protein expression in the eye.

Besides, an insertion/deletion variant between the *ARMS2* and the *HTRA1* genes significantly induces *HTRA1* transcription regulator activity in photoreceptor cell lines, and the insertion/deletion variant region should be potentially surrounded by transcriptional suppressors and activators [63]. Liquid chromatography-mass spectrometry identified the LYRIC (lysine-rich CEACAM1 co-isolated) protein binding to the insertion/deletion region. In addition, induced pluripotent stem cells from neovascular AMD patients carrying the insertion/deletion variant showed significant upregulation of *HTRA1* transcript compared to the controls. Whether the insertion/deletion variant is in the same risk haplotype with the rs11200638 and rs2672598 variants requires further fine mapping analysis. Collectively, the cis-regulatory variants in the *HTRA1* promoter region likely induce the upregulation of HTRA1 expression. High *HTRA1* expression induces RPE cell death [64], resembling the pathological changes in AMD development.

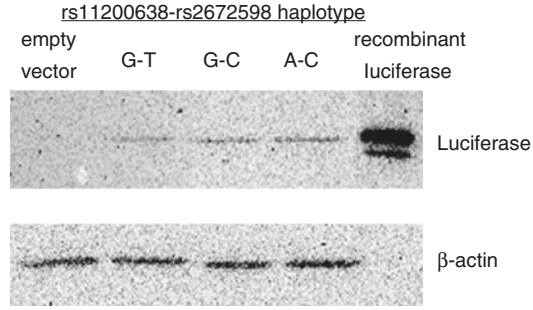

Fig. 19.2 Luciferase expression analysis on the *HTRA1* promoter of the rs11200638-rs2672598 haplotype. Detection of luciferase expression was performed by immunoblotting. The wild-type rs11200638-rs2672598 haplotype (G-T) is the wild type reference. Comparing to the G-T haplotype, elevated luciferase expression was observed for the G-C and A-C haplotypes, indicating that the C-allele of rs2672598 variant enhances the transcription activity of *HTRA1* promoter. In contrast, there was no difference in luciferase expression level between the G-C and the A-C haplotypes, suggesting that the A-allele of rs11200638 would not alter the transcription activity of *HTRA1* promoter. Recombinant firefly luciferase was used as a positive control, whereas empty pGL3 vector was used as a negative control

19.3.3 Tumor Necrosis Factor Receptor Superfamily Member 10A Gene

Tumor necrosis factor receptor superfamily member 10A (*TNFRSF10A*)-*LOC389641* on chromosome 8p21 was first identified as a susceptible locus for neovascular AMD in the Japanese population [65]. The most significantly associated variant (rs13278062: T>G) is located in the promoter region of *TNFRSF10A* gene. In collaboration with the Kyoto Japanese cohort, we validated the association of *TNFRSF10A* promoter variant rs13278062 with neovascular AMD in the Asian

population [66]. However, the association of rs13278062 variant with neovascular AMD was not identified in the Beijing Chinese cohort [67]. A meta-analysis showed a nominal association of *TNFRSF10A* promoter variant rs13278062 with an increased risk of advanced AMD (OR = 1.17). The *TNFRSF10A* promoter variant rs13278062 is also significantly associated with the second-eye involvement in the Japanese population [68]. In addition, the recent large GWAS analysis also identifies the significant association of another *TNFRSF10A* promoter variant rs79037040 with AMD [46], indicating that *TNFRSF10A* expression level variation should be involved in the pathogenesis of AMD.

Although the contribution of gene to the AMD development could be minor (OR = 0.7–0.9), the activator protein 1 binds to the region around rs13278062 and regulates *TNFRSF10A* gene expression [69]. It has been reported that the G-allele of *TNFRSF10A* promoter variant rs13278062 enhances the transcription activity of *TNFRSF10A* promoter when compared to the wild-type T-allele [70]. *TNFRSF10A* gene encodes for TRAIL receptor 1 (TRAILR1), also known as death receptor 4, which is broadly expressed in human RPE and mouse rod photoreceptors [71]. Activation of TRAILR1 can induce apoptosis through caspase-8 pathway [72] as well as the production of inflammatory cytokines and the promotion of inflammation through NF-κB pathway [73]. Dysregulation of *TNFRSF10A* gene expression could be involved in the pathogenesis of AMD.

19.3.4 Lipase C Gene

Lipase gene (*LIPC*, hepatic type) on chromosome 15q21.3 was first identified to be associated with AMD by GWAS analysis in the Caucasian population, which the AMD-associated variants (rs493258 and rs10468017) are located in the promoter region of LIPC gene [74]. The association of *LIPC* promoter variants rs493258 and rs10468017 with advanced AMD is confirmed in two independent Caucasian populations [75]. However, the rs10468017 variant is not associated with advanced AMD in the Indian population [76]. Nevertheless, there could be a possible interaction among *LIPC* rs10468017 variant, *CFH*, and complement factor I (*CFI*) variants in AMD risk prediction [77].

The minor T-allele of *LIPC* rs10468017 variant, with a reduced risk of AMD (OR = 0.4–0.5), reduces the expression of LIPC gene [74], and it is associated with higher levels of serum high-density lipoprotein (HDL; [78]), Although there is a lack of consistent association between HDL alleles and AMD risk, the LIPC and HDL effects could be indirect and accumulative. Changes in HDL-mediated transport of lutein and zeaxanthin could be a possible mechanism by variations in LIPC levels to the risk of AMD [79]. Furthermore, drusen, the hallmark of AMD, also contain cholesterol deposits [80], indicating an aberrant in cholesterol transport. Yet, there are no significant interactions between LIPC and smoking, body mass index (BMI), or lutein [77].

19.3.5 Other Genes

Vascular endothelial growth factor A (*VEGFA*) gene locus on chromosome 6p12 was first confirmed to be associated with advanced AMD in the Caucasian populations by GWAS analysis [81]. Although the *VEGFA* promoter variant rs699947 (A>C) shows no significant association with AMD [82], the C-allele of *VEGFA* rs699947 variant is associated with higher VEGF production [83]. Instead, the C-allele of *VEGFA* rs699947 variant is correlated with better response to ranibizumab treatment than the A-allele in multiple populations [84, 85]. In contrast, the C-allele of *VEGFA* rs699947 variant is significantly higher in photodynamic therapy (PDT) nonresponders than the PDT responders in the Finland population [86].

Interleukin-8 (*IL8*) promoter variant rs4073 (c.-251A/T) was first reported to be associated with AMD in the British population by a candidate gene analysis [87]. This promoter variant is confirmed to be associated with younger onset age of neovascular AMD in the Finland population [88]. Moreover, the *IL8* promoter variant

rs4073 is also associated with persisting fluid in optical coherence tomography [89]. The A-allele of rs4073 variant is more frequent in nonresponders of initial bevacizumab treatment than in responders, and it can predict poorer outcome together with the occult or predominantly classic lesions. The A-allele of *IL8* promoter variant rs4073 is associated with higher levels of circulating and secreted IL-8 protein [90]. Higher IL-8 production could lead to IL-8 stimulated angiogenesis and capillary leakage [91].

Apolipoprotein E (*APOE*) variant (rs2075650) on chromosome 19q13.32 was first suggested to be associated with early AMD by a GWAS meta-analysis [92]. The large GWAS analysis also confirms the significant association of APOE variant (rs429358) with AMD [46]. However, the APOE ε4 genotype is not associated with AMD in our Hong Kong Chinese population [93]. Yet, the extended haplotype analysis demonstrated a significant association of APOE haplotype, including an *APOE* promoter variant rs405509 (G>T), with AMD [94], suggesting that the relative rate of APOE isoform expression would be crucial in AMD pathogenesis based on the influence of *APOE* promoter activity by the rs405509 variant [95]. However, a pooled analysis of 15 studies indicated that the extended haplotype with rs405509 variant does not increase additional risks beyond the ε2 and ε4 haplotypes [96].

Excision repair 6, chromatin remodeling factor (*ERCC6*) promoter variant c.-6530C>G was first reported to be associated with AMD and interact with *CFH* variant rs380390 in the Caucasian population [97]. The putative transcription factor binding site is predicted to be changed in the G-allele of *ERCC6* promoter variant, and the luciferase expression is higher in the G-allele of *ERCC6* promoter variant compared to the wild-type C-allele. Intense ERCC6 expression was also found in AMD eyes with the G-allele of *ERCC6* promoter variant. Another *ERCC6* promoter variant rs3793784 was reported to confer a small increase in risk for advanced AMD in the Dutch populations, but not replicated in two non-European cohorts [98]. In contrast to the c.-6530C>G variant, early AMD-affected donor eyes showed lower ERCC6 expression than healthy donor eyes. Whether increase or decrease in *ERCC6* transcriptional activity contributing to the AMD development requires further investigations.

Serpin family G member 1 (*SERPING1*) variant rs2511989 on chromosome 11q12.1 was first reported to be associated with AMD in the British population by low-density variant screening [99]. The *SERPING1* variant rs2511989 is not associated with AMD in our Hong Kong Chinese population as well as other East Asian populations, but associated with AMD in the Caucasian populations [100]. *SERPING1* gene encodes the C1 inhibitor, which is crucial in inhibiting the complement component 1 (C1) in the classic complement pathway. Although the *SERPING1* promoter variant rs2649663 is not associated with AMD, it is associated with C1 inhibitor levels and higher level of C1 inhibitor was shown in AMD patients compared to the control subjects in the British population [101]. This suggests that *SERPING1* promoter variation could also influence the expression of *SERPING1* gene.

Matrix metalloproteinase-2 (*MMP2*) variant rs2287074 has been shown to be associated with AMD, and. the A-allele is associated with a lower likelihood of AMD in older Caucasian women [102]. An *MMP2* promoter variant rs243865 (c.-1306C>T) was reported to be associated with AMD in the northern Chinese population [103]. However, no association of *MMP2* promoter variant rs243865 with AMD was observed in the Turkish and Lithuania populations [104, 105]. Instead, the *MMP2* promoter variant rs243865 is associated with younger AMD onset in male patients [106]. Besides, the plasma levels of MMP-2 in AMD patients are not significantly different from that of the control subjects [107], indicating that MMP-2 is unlikely play a major role in the pathogenesis of AMD.

Toll-like receptor 3 (*TLR3*) variant rs3775291 on chromosome 4q35.1 was first reported to be associated with non-neovascular AMD in the Caucasian population [108]. However, the *TLR3* promoter variants rs5743303 and rs5743305 are not associated with neovascular AMD in the northern Chinese population [109].

Mice deficient with CC-cytokine ligand 2 (*Ccl2*) gene, also known as monocyte che-

moattractant protein-1, develop the pathological features of AMD, including accumulation of lipofuscin in RPE, the presence of drusen beneath RPE, photoreceptor atrophy as well as CNV [110]. However, the promoter variants c.-2578A>G and c.-2136A>T of CCL2 gene are not associated with AMD in the Netherlands Caucasian population [111].

19.4 Glaucoma

Glaucoma is the leading cause of irreversible blindness and visual impairment, which would affect 76 million people worldwide in 2020 [112]. Primary glaucoma can be subclassified into primary congenital glaucoma, primary open angle glaucoma (POAG), and primary angle closure glaucoma (PACG). They share common pathologies of retinal ganglion cell loss and the axonal degeneration. Although research studies have deciphered most of the glaucoma pathogenesis, elevated intraocular pressure (IOP; >21 mmHg) is the only recognized modifiable risk factor in glaucoma treatment, which the progression of glaucoma can be attenuated when the IOP is lowered by 30–50% [113]. Yet, normal intraocular pressure can also be found in a number of POAG patients [114]. Nevertheless, the IOP lowering treatment is the only proven treatment for all forms of glaucoma [115].

The inheritance of glaucoma has been suggested for 70 years [116]. Earlier studies relied on family linkage analysis to map the disease genes/loci for glaucoma in large pedigrees [117, 118]. Similar to AMD, the discovery of glaucoma-associated genes has been boosted with the application of GWAS. The first GWAS-identified glaucoma gene is the lysyl oxidase-like 1 (*LOXL1*) gene for exfoliation glaucoma in the Icelandic population [119], whereas the first POAG GWAS identified 3 susceptible loci in the Japanese population [120]. Moreover, there are 3 GWAS analyses on PACG, mainly based on the Asian populations [121–123]. Most of the GWAS-identified variants are located in the intergenic region, indicating the possible involvement of the transcriptional regulation on the disease-associated gene expression. In this section, the promoter variants for glaucoma were summarized and discussed.

19.4.1 Myocillin Gene

MYOC on chromosome 1q24.3 is the first disease-causing gene identified for POAG [38]. Its mutations account for 0.3–4.3% of POAG patients [124]. Apart from the mutations in exons, a *MYOC* promoter variant mt.1 (–1000 C/G) is associated with more rapid worsening for both optic disc and visual field measures of glaucoma progression [125]. It is also associated with poor IOP control, greater visual field damage, and a lack of response to therapeutic intervention in POAG patients [126]. However, in our Hong Kong Chinese population, the *MYOC* mt.1 promoter variant is not associated with the risk of POAG [127]. In addition, a meta-analysis showed that another *MYOC* promoter variant rs2075648 is significantly associated with POAG risk in the Caucasian populations, but not in other ethnic populations [128]. These indicate that the association of *MYOC* promoter variants with POAG could be specific in the Caucasian populations.

19.4.2 Cytochrome P450 Family 1 Subfamily B Member 1 Gene

Cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*) gene on chromosome 2p21 was identified as the disease-causing gene for primary congenital glaucoma [129]. Similar to the *MYOC* gene, *CYP1B1* promoter variant rs2567206 (c.-236T>C) has been reported to be associated with primary congenital glaucoma in the Indian population, but not with POAG and PACG [130]. Luciferase assay in the trabecular meshwork cell line showed a 90% reduction in *CYP1B1* promoter activity with the C-allele of rs2567206 variant, compared to the T-allele. However, a meta-analysis of six studies reported no significant association of *CYP1B1* promoter variant rs2567206 with POAG [131].

19.4.3 Caveolin-1 Gene

Caveolin-1 (*CAV1*)/*CAV2* locus on chromosome 7q31.2 was first identified to be associated with POAG in the Icelandic population by GWAS analysis [132]. The most significantly associated variant rs4236601 is located in the promoter region of *CAV1* gene. We confirmed the association of CAV1 rs4236601 variant with POAG in the northern and southern Chinese populations with OR of 5.26; however, this variant is not polymorphic in the Osaka Japanese cohort [133]. In spite of its association with POAG, the genotypes of rs4236601 would not influence the expression and distribution of CAV1 protein in the retinas of donor's eyes from the Caucasian population [134]. Apart from the rs4236601 variant, another variant located upstream of the *CAV1* gene (rs17588172:T>G) was also shown to increase 1.5-fold susceptibility to high tension glaucoma and associated with IOP elevation in the Korean population [135]. It is also associated with early paracentral visual field in POAG patients [136]. The G-allele is associated with the decreased *CAV1* gene expression in skin and adipose by the Genevar eQTL analysis [135]. Coherently, we demonstrated that CAV1-knockout weakens the adhesion of human trabecular meshwork cells and increases the autophagy activity (Wu et al. unpublished data). Collectively, the reduced *CAV1* expression could contribute to the development of POAG.

19.4.4 Cyclin-Dependent Kinase Inhibitor 2B Gene

Cyclin-dependent kinase inhibitor 2B (*CDKN2B*) gene variant (rs1063192) on chromosome 9p21 was first identified to be associated with the vertical cup-disc ratio in a GWAS analysis on the optic disc parameters [137]. In the Australian population, one CpG island (F1:13-14) in the *CDKN2B* promoter showed a significant association with normal tension glaucoma, especially in female subjects [138]. The methylation at the CpG islands in the *CDKN2B* promoter is also associated with genotype at rs1063192, indicating that the expression variation of *CDKN2B* gene could be involved in the development of POAG.

19.4.5 Lysyl Oxidase-Like 1 Antisense RNA 1 Gene

LOXL1 gene on chromosome 15q24.1 is the first GWAS-identified gene for exfoliation glaucoma [119]. Instead of the *LOXL1* gene variant, the variants in the LOXL1 antisense RNA 1 (*LOXL1-AS1*) gene promoter region, the long noncoding RNA encoded on the opposite strand of *LOXL1*, showed strongest association with exfoliation syndrome in the South African population [139]. The *LOXL1-AS1* expression could be changed in response to oxidative stress in human lens epithelial cells and in response to cyclic mechanical stress in human Schlemm's canal endothelial cells. The variants in the *LOXL1-AS1* promoter region could modulate the activity of the *LOXL1-AS1* promoter, which could contribute to the development of exfoliation glaucoma.

19.4.6 Apolipoprotein E Gene

The Alzheimer's disease-associated *APOE* promoter variants were first suggested to be associated with the POAG phenotypes by the candidate gene analysis [140]. The *APOE* promoter variant (c.-219G>T) is associated with the increased cup-to-disk ratio and visual field alteration, whereas the c.-491A>T variant interacts with the *MYOC* promoter variant (-1000 C/G) and is associated with increased IOP and poor response to the IOP-lowering treatments in POAG patients. In the British population, no evidence of association between *APOE* promoter variants c.-219G>T or c.-491A>T and POAG was found [141]. In the Turkish population, although the *APOE* promoter variant (c.-219G>T) showed no significant association with POAG, the POAG patients carrying the GG genotype have higher mean linear cup-to-disc ratio and disease progression, compared to those carrying the GT genotype [142]. Similarly,

in our Hong Kong Chinese population, no significant difference was detected in the frequencies of *APOE* promoter variants between POAG patients and control subjects [143]; yet, the POAG patients with the G-allele of c.-219G>T variant carriers showed a higher age of diagnosis compared to those with the TT genotype. Altogether, these indicate that the *APOE* promoter variants could be a potent modifier for POAG.

19.4.7 Inflammation-Related Genes

The tumor necrosis factor-α (*TNFA*) promoter variant (c.-308G>A) is associated with POAG and pseudoexfoliation glaucoma, but not with chronic PACG in the Iran population [144]. It is also associated with POAG in the Turkish population [145]. However, a meta-analysis of 13 studies revealed no significant association of the *TNFA* c.-308G>A variant with any type of glaucoma [146]. This meta-analysis also showed no significant association of the *TNFA* c.-238G>A variant with glaucoma. Instead, the A-allele of the *TNFA* c.-863C>A variant is lower in POAG patients from the Taiwan population, compared to that in control subjects [147]. Besides, The frequency of (T-allele of *TNFA* c.-857C>T variant and A-allele of optineurin (*OPTN*) c.412G>A variant) or (A-allele of *TNFA* c.-863C>A variant and A-allele of *OPTN* c.603T>A variant) carriers is significantly higher in POAG patients than in control subjects from the Japanese population [148]. These carriers had significantly worse visual field scores than those without *OPTN* variants.

The *IL1A* promoter variant (c.-889C>T) showed an increased risk to POAG in the Taiwan population [149]. The T-allele of the *IL1A* c.-889C>T variant has been shown to increase the expression of IL1A gene. In contrast, the *IL1B* promoter c.-511 is not associated with POAG in the Taiwan population [150]. Besides, the *IL6* promoter variant c.-174G>C has also been reported not to be associated with POAG in the Austrian population [151].

19.4.8 Nitric Oxide Synthase Genes

The endothelial nitric oxide synthase (*NOS3*) promoter variant (c.-690C>T), lying between the cAMP regulatory element (c.-726 to c.-732) and an activator protein-1 binding domain (c.-655 to c.-661), is significantly associated with familial POAG [152]. However, the *NOS3* promoter variant (c.-786T>C) is not associated with POAG in the Taiwan Chinese population [153]. Instead, the normal tension glaucoma patients with CC genotypes of the *NOS3* c.-786T>C variant showed lower mean diastolic and systolic pressure during the day and night in the Poland population [154].

The CCTTT-microsatellite in the inducible nitric oxide synthase (NOS_2) gene promoter showed a significant difference in allele distribution between POAG patients and control subjects in the Sweden population [155]. The (CCTTT)14 allele, which is significantly more abundant in POAG patients, exhibits specific binding of nuclear proteins and a higher reporter activity.

19.4.9 Matrix Metalloproteinase Genes

A meta-analysis of five studies with 1261 glaucoma patients and 1089 control subjects showed a significant association of *MMP1* promoter variant rs1799750 with PACG under homozygous and allelic models and with POAG and exfoliation glaucoma under recessive model [156].

The *MMP2* promoter variants c.-735C>T and c.-1306C>T are not associated with POAG; yet, the TT genotype of both *MMP2* promoter variants are significantly associated with the rim area factor at the early stage of POAG patients from Poland [157].

The *MMP9* promoter variant c.-1562C>T is significantly associated with POAG and PACG under the dominant model in north Indian population [158]. The T-allele of the *MMP9* c.-1562C>T variant confers 1.9-fold higher risk of developing PACG for male patients as compared to the control subjects.

19.4.10 Other Genes

The catalase (*CAT*) promoter variant rs1001179:C>T showed a trend of increase in the visual acuity of PACG patients in the Saudi Arabia population, compared to the control subjects [159].

19.5 Cataract

Cataract remains the leading cause of reversible blindness in developing countries, affecting 95 million people worldwide [160]. Based on the etiology, cataracts can be classified as age-related cataract, pediatric cataract, and secondary cataracts. Age-related cataract is most common in adults, with the onset between age 45 and 50 years. Even with the advancement of technologies and techniques for cataract surgery, the pathogenesis of age-related cataract remains elusive, which is believed to be greatly influenced by the environmental factors. Congenital cataract refers to lens opacity presented at birth, whereas infantile cataract refers to lens opacity developed during the first year of life. Pediatric cataracts have a different pathogenesis than that of age-related cataracts.

Cataract genetic research studies focused on pediatric cataract as one-third of pediatric cataracts are inherited [161]. With the development of whole exome sequencing analysis [162], more than 1000 gene variants have been identified for inherited cataracts in family linkage and candidate gene studies (https://cat-map.wustl.edu/; [163]). Compared to the congenital cataracts, the genetic variants contributing to age-related cataract are largely unknown, which could be complicated by the influences of environmental factors, including sunlight exposure and cigarette smoking [164]. Nevertheless, a recent GWAS analysis on 7050 patients with age-related nuclear cataract identified two loci for nuclear cataract: *KCNAB1* and *CRYAA* [165]. In this section, the promoter variants for cataracts were summarized and discussed.

19.5.1 Crystallin-α A Gene

Crystallin-α A (*CRYAA*) gene, a major protein component of lens, on chromosome 21q22.3 was first identified for the autosomal dominant congenital cataract [166]. A variant (rs11911275) downstream of *CRYAA* gene was also reported to be associated with age-related nuclear cataract in Asian populations, which the downregulation of CRYAA in human lens capsule is correlated with the increase severity of nuclear cataract [165]. In addition to the downstream variant, 2 *CRYAA* promoter variants (rs13053109 and rs7278468) were also reported to be associated with age-related cataract as well as cortical cataract [167]. The rs7278468 variant lies in a consensus binding site for the transcription repressor KLF10, and the T-allele of rs7278468 variant is associated with the increased binding of KLF10 and the inhibition of *CRYAA* transcriptional activity. The epigenetic repression of *CRYAA* gene has been implicated in age-related cataract [168] as well as in high-myopic cataract [169].

19.5.2 Crystallin-γ B Gene

Crystallin-γ B (*CRYGB*) mutation on chromosome 2q33.3 is rare for congenital cataract [170]; yet, the *CRYGB* promoter variant rs2289917 (c.-47T>C), which is predicted binding to ACE2 and progesterone receptor transcription factors, varies significantly among different age groups in the control population of western Indian origin [171]. The C-allele of *CRYGB* rs2289917 variant confers an increase susceptibility to pediatric cataract with OR of 3.34 in the Indian population [172]. In addition, the *CRYGB* rs2289917 variant is also associated with age-related cataract in the Ukrainian population, and the patients with CC genotype of the rs2289917 variant showed higher expression of CRYGB in platelets, compared to those carrying the T-allele [173].

19.5.3 Ferritin Light Chain Gene

Ferritin light chain (*FTL*) gene on chromosome 19q13.33 was discovered for the autosomal dominant trait of hereditary hyperferritinemia-cataract syndrome with a combination of elevated serum ferritin not related to iron overload and congenital nuclear cataract [174]. Point mutations, such as c.-176T>C, c.-171C>G, c.-168G>T, c.-167C>T, and c.-161delC [175–177] were found in the cis-acting element of *FTL* promoter, known as iron regulatory element (IRE). The mutations in the IRE disturb the binding of iron regulatory proteins, leading to an increase in FTL production regardless of the serum iron concentration [178].

19.5.4 Transmembrane Protein 114 Gene

Transmembrane protein 114 (*TMEM114*) gene on chromosome 16p13.2 was discovered as the disease-causing gene for congenital lamellar cataract because of a balanced familial chromosomal translocation t(16;22)(p13.3;q11.2) [179]. The breakpoint lies in the promoter region of *TMEM114* gene and separates this gene from the predicted eye-specific upstream transcription factor binding sites. Further mutation screening in congenital cataract patients identified missense mutations (p.I35T and p.F106L) in *TMEM114* gene, confirming its contribution to congenital cataract. In the mouse lens, Tmem114 expression was found in the lens epithelial cells extending into the transitional zone, possibly involved in early fiber differentiation.

19.5.5 Ras Related GTP Binding A Gene

Ras related GTP binding A (*RRAGA*) gene on chromosome 9p22.1 was discovered to be associated with autosomal dominant juvenile-onset cataract in our Shantou Chinese cohort by whole exome sequencing analysis [180]. In addition to the missense mutation (p.Leu60Arg), we identified a promoter variant (c.-16G>A) of the *RRAGA* gene in a patient with congenital nuclear cataract. This c.-16G>A promoter variant was predicted to abolish a CpG island and a binding site for E2F1, a transcription factor that regulates mechanistic rapamycin complex 1 (mTORC1) signaling. Luciferase reporter assay confirmed that the A-allele of the c.-16G>A promoter variant showed lower transcription activity than the G-allele.

19.5.6 Other Genes

The interferon-γ receptor 1 (*IFNGR1*) promoter variant (c.-56C>T) was reported to be associated with an increased risk of atopic cataracts in the Japanese population [181]. The reporter assay showed that, after stimulation with IFN-γ, the T-allele of the c.-56C>T variant showed higher transcriptional activity of *IFNGR1* gene in lens epithelial cells than the C-allele. Furthermore, higher *IFNGR1* gene expression was found in lens epithelial cells with atopic cataract, compared to that in senile cataracts.

Ephrin receptor A2 (*EPHA2*) gene has been shown to be associated with childhood cataract as well as age-related cataract [182]. A *EPHA2* promoter variant rs6603883, lying in a PAX2 binding site, showed a decreased EPHA2 transcriptional activity in the C-allele, compared to the T-allele, by reducing the binding affinity of PAX2 [183].

Although the catalase (CAT) activity has been shown to be reduced in the plasma of cataract patients than that in the control subjects [184], the *CAT* promoter variant (c.-21A>T) is not significantly associated with age-related cataract in the Chinese population [185]. Coherently, another *CAT* promoter variant c.-262C>T is also not associated with the risk of age-related cataract in the Iran population [186].

19.6 Summary and Future Perspectives

The contribution of promoter variants to the promoter activity and the gene expression is clear and definite. Investigations on the association of promoter variants with complex eye diseases are challenging: (1) Genetic variants exist in a haplotype with strong linkage disequilibrium. It is difficult to identify the causal variant just based on the statistical methods. The localization of the risk and protective variants could provide a hint, which the causal risk variant would not locate in the same allele with another protective variant [64]. Nevertheless, it is still a rate-limiting step for the functional analyses on each variant. (2) Limited studies were reported to study a particular promoter variant with complex eye diseases, and the association of the promoter variants with the complex eye diseases could be population specific. Replication studies in different populations should be conducted to verify the association of each individual promoter variant. (3) Misexpression of a gene is not only caused by the promoter variants with transcription factor binding site changes, but could also be affected by multiple processes, including copy number variation [187] as well as stability and subcellular localization of mRNA and protein [188]. (4) Transcriptomics is a dynamic process. Single variant in the promoter region might not solely contribute to the disease phenotypes. Interactions with other variants or other genes could be possible, but complicated the whole scenario. The retinal cells derived from the induced pluripotent stem cells carry the patients' genome and could mimic the transcriptome of the patients' cells [189]. (5) How the promoter variants can cause the complex eye diseases? Further research is needed to understand the underlying mechanisms of long-term, low-dose aberrant gene expression in the development of complex eye diseases.

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20 Vascular Basement Membrane Thickening: Basis of Disease Pathology in Diabetic Retinopathy

Aravind Sankaramoorthy and Sayon Roy

Abstract

Diabetic retinopathy (DR) is one of the most common forms of microvascular complications of diabetes. Unfortunately, there is no cure for this debilitating ocular complication that holds particular significance due to its detrimental effects on vision, which ultimately leads to vision loss and blindness. Hyperglycemia, the most prominent characteristic of diabetes, plays a major role in initiating the development and progression of DR. One of the histological hallmarks of the pathogenesis of DR is characterized by the thickening of the basement membrane (BM). Abnormalities in the BM ultrastructure affect retinal vascular cell attachment and compromises the inner blood–retinal barrier. While strides are being made to better understand the cellular events regulating retinal capillary BM thickening, including high glucose-induced upregulation of type IV collagen, fibronectin, and laminin genes together with decreased matrix metalloproteinase (MMP) activities, and its role in the pathogenesis of DR, the focus is now to apply effective strategies to prevent BM thickening to establish its therapeutic potential.

Keywords

High glucose · Fibronectin · Collagen IV
Endothelial cells

20.1 Introduction

20.1.1 Structure and Biological Function of the BM

The term "basement membrane" was initially coined and recognized by Sir William Bowman as a homogenous membranous layer surrounding the cells of the epithelium, mesenchyme, and the endothelium [1]. BM refers to an extension of the cell boundaries from the plasma membrane, forming an extracellular matrix (ECM) that creates an interface between cells and the surrounding environment [2]. In general, the BM is composed of proteins that are secreted by the underlying cells (endothelium, epithelium, and smooth muscle cells) [3]. BMs are continuous sheets of ECM material composed of collagenous and non-collagenous glycoproteins connecting the cellular boundaries to the ECM. These glycoproteins are also produced by vascular cells, endothelial

A. Sankaramoorthy · S. Roy (✉)
Department of Medicine, Boston University School of Medicine, Boston, MA, USA

Department of Ophthalmology, Boston University School of Medicine, Boston, MA, USA
e-mail: aravind.sankaramoorthy@bmc.org; sayon@bu.edu

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cells [4], and pericytes [5], located in the retinal microvessels [6]. Before electron microscopy came into vogue, vascular BM was referred to as a Periodic Acid-Schiff (PAS)-positive region surrounding capillaries that could be seen under the light microscope. Electron microscopic examination of the vascular BM revealed a moderately electron-dense layer present between endothelial cells and pericytes [7]. Some studies indicate a further subdivision of the BM as lamina densa facing the stroma, and lamina lucida facing the cell or the plasma membrane [8]. However, these layers within the BM are not always clearly demarcated. Thus, the BM of small blood vessels is lined with cells on both surfaces; endothelial cells on the luminal side and pericytes on the abluminal side. They act as mechanical support structures for the attachment of cells and define tissue boundaries. The semipermeable barriers between the tissues regulate cellular migration and differentiation [8].

ECM has played a major role in the evolution of multicellularity of animals. Many components of the ECM, such as laminin and type IV collagen, were acquired from common ancestors of metazoans [9]. Since then the BM structure continued to evolve, attaining specific functionality within different tissues including those of the microcirculation [10]. The supramolecular structure of the BM is made up of several proteins that are organized in a specific manner creating a continuous sheet of ECM that is composed of laminin, type IV collagen, fibronectin, and heparan sulfate proteoglycans [11].

20.1.2 Structure and Components of the BM

Fibronectin (FN) Fibronectin is an important component of the BM and also an adhesion protein, which anchors the cells to other components of the ECM. It also plays a major role in maintaining the structural integrity of the BM, cell adhesion, migration, differentiation, and growth. It exists as a homodimer containing identical 250 kDa subunits, which are linked at their C-terminal region by disulfide bonds. The blood plasma contains FN in a soluble protomeric form while the ECM is composed of an insoluble multimeric form [12–15].

Collagen IV Collagen IV is often referred to as the BM collagen, and one of the most widely distributed [16] and major component of the BM [17]. Collagen IV is a highly conserved protein and has played a significant role in the process of evolution of multicellular organisms [9]. The expression of collagen IV is tightly regulated in a tissue-specific manner, and is encoded by COL4A1–COL4A6 genes. Type IV collagen is a triple-helical structure with two α1 chains and one α2 chain. The assembly of collagen IV monomers to form a mature triple helical structure is very critical for the proper functioning of the protein and maintenance of the BM framework. Collagen IV has a globular (NC1) domain in the COOH terminus and an amino terminal (NH_2) region known as a 7S domain [18]. Three α chains are assembled to form a triple-helical structure intracellularly forming the collagen IV protomer, which is then transported to the extracellular region where two protomers bind through their carboxy terminal (NC1) regions to form a hexamer. A mature collagen IV molecule of the BM consists of four hexamers, which bind via the 7S or the amino terminal region [19, 20].

Laminin Laminin is a multimeric non-collagen ous, glycoprotein (≥800 kDa) made up of three polypeptide chains (A, B1, B2) connected by disulfide bonds [21–23]. The electron microscopy structure of laminin reveals an asymmetric four-arm structure with three short arms of about 35 nm B-chains and a 50 nm A-chain, while the long arm is about 75 nm. The three short arms of laminin are formed by one of the three polypeptide chains (A, B1, B2) and these three polypeptide chains extend to form the long arm with an extended globule formed by the A chain. The short arms mediate the self-assembly and the C-terminal globule of the long arm binds to heparin [24].

Heparan Sulfate Proteoglycan Heparan sulfate proteoglycans (HSPGs) are single polypeptide chains which are about 400–500 kDa in size and 80 nm in length [25, 26], and form an important component of most BMs. The single polypeptide chain forming the core is substituted by three heparan sulfate chains of 32 nm regions [24, 26]. HSPGs prevent proteolytic degradation of certain proteins, such as growth factors, chemokines, cytokines, and morphogens, and therefore serve as a store of regulatory factors. They also mediate cell differentiation, and leukocyte recruitment and storage by creating a morphogen gradient and a chemokine gradient, respectively. Proteoglycans present in the membranes coordinate cell motility, cell–cell, cell–ECM interactions via integrins [27].

20.1.3 Other BM Components

Nidogen Nidogen, also known as entactin, is found in all BMs and is 158 kDa in size, which contains a single chain glycoprotein [28]. Electron microscopy analysis revealed a dumbbell-shaped structure containing two terminal globular domains (5.8 and 4.5 nm), which are connected to a 17-nm rod-like structure [29, 30]. There are two isoforms of nidogen in mammals, namely nidogen-1 and nidogen-2. Structurally, they are elongated molecules having three globular domains (G1, G2, and G3) [31]. One of the major functions of nidogen is linking the laminin to collagen IV networks during the BM assembly [32, 33]. The role of nidogen as a cell adhesion molecule is still under investigation, however, evidence show that they mediate cell attachment. Nidogen binds to the short arm of laminin in the intersection of the region where the binding sequence YIGSR [34] of the B1 chain and RGD of the A chain is present [35]. The presence of nidogen in the cell-binding region of laminin could modulate the cell-binding properties of laminin, thereby regulating the matrix and cell attachment process [36]. In DR, nidogen-1 is found to be upregulated in the retinal vascular BM of streptozotocin (STZ)-induced diabetic mice [37]. Nidogen has been shown to be critical for the proper assembly of BM, as loss of nidogen during the BM assembly results in fatal developmental abnormalities in multiple organs after birth [38].

Vitronectin Vitronectin is a component of the ECM, which is a 75-kDa glycoprotein, with three glycosylation sites and a carbohydrate moiety which makes up 35% of its total molecular mass [39]. The amino terminal region of vitronectin contains amino acid sequences similar to somatomedin B, which binds the plasminogen activator inhibitor-1 [40]. It also harbors the Arg-Gly-Asp (RGD) domain, which mediates the migration and attachment of cells to their ECM through receptors in the integrins. Vitronectin also binds to the collagen in the BM via regions adjacent to the RGD sequence and the heparin-binding domain [40, 41]. Interestingly, an increase in the plasma and vitreous levels of vitronectin has been reported in patients with type II diabetes with PDR, which may result in the angiogenic growth of endothelial cells in the retinal vasculature [42].

SPARC SPARC, or osteonectin, is a 32-kDa secretory glycoprotein involved in remodeling of the BM, angiogenesis, and cell proliferation [43]. Recent studies have revealed that SPARC binds to the triple helical structure of type IV collagen in a Ca^{2+}-dependent manner in mouse EHS recombinant human SPARC, suggesting that SPARC may be involved in providing cell anchorage to the BM [44]. SPARC is believed to have a role in the BM as studies on endothelial and smooth muscle cells reveal that SPARC mediates the modulation of shape of the underlying endothelial cells, smooth muscle cells, and fibroblasts [45, 46]. Additionally, increased SPARC expression has been reported in vitreous samples from patients with PDR [43].

20.1.4 Assembly of BM Components

BM components are capable of self-assembly as each of the components harbors the information for binding to the specific sites on other macromolecules. The assembly of BM is a multistep process and is initiated by the binding of laminin to the cell surface via integrins [47–51]. The binding of laminin and type IV collagen polymerization creates a scaffold of matrix on which the other components are assembled in a supramolecular architecture [49, 50, 52]. The primary interaction is initiated by the binding of laminin LG domain to integrins, sulfated glycolipids, dystroglycan, and heparan sulfates. The assembly of BM is a carefully regulated process, which involves a balance of the synthesis and accumulation of BM components and receptor expression and the degradation of the BM by MMPs. Furthermore, the overall organization of the BM size in terms of density can also be altered by changes in cytoskeleton through receptor connections [53].

20.1.5 BM Stiffness

Mechanical stiffness of BM is important in understanding the signaling between the cells and the ECM, as well as between endothelial cells and pericytes. These signals involve changes in gene expression and overall regulate the function of the cellular machinery [54], including those pertaining to vascular dysfunctions [55] and endothelial cell loss [56]. Interestingly, the role of lysyl oxidase (LOX), a crosslinking enzyme of type IV collagen, and its upregulation in DR have been shown to increase BM stiffening, which results in inflammation [57]. The coordinated effect of increased ICAM expression and the adhesion of monocyte to the endothelial lining [58, 59], along with increased expression of type IV collagen and LOX play a significant role in promoting capillary BM stiffness [57].

20.2 Biological Function of the BM

20.2.1 Selective Permeability Barrier

Selective permeability of molecules is important to maintain homeostasis, and the BM plays a significant role in regulating the selective permeability process. In the vasculature, the inner layer of microvessels is occupied by endothelial cells, which overlay a thin layer of basal lamina. The BM prevents growth factors, hormones, and polysaccharides from leaking out of the bloodstream into the adjacent connective tissue. In the capillary endothelial BM, the tight junctions that are formed between the endothelial cells in the paracellular region also regulate permeability. Type IV collagen and laminin are necessary for the proper functioning of tight junctions, indicating that BMs are critical in maintaining the retinal capillary permeability characteristics [60].

20.2.2 Substratum for Cell Attachment

BM's primary role is to provide a substratum for cell attachment and also acts as a physical barrier between different cell types and tissues, thereby maintaining any changes in cell shape or size [61]. The assembly of the components and their supramolecular architecture is very important for cell attachment. Type IV collagen [62], laminin, fibronectin, and nidogen all play a crucial role in cell attachment, as downregulation of these BM components has been shown to decrease cell attachment [36].

20.2.3 Apoptosis

BM plays a major role in regulating cell survival and cell proliferation depending on the signaling of growth factors, adhesion proteins, and the

components of the BM [63, 64]. Moreover, BM proteins such as laminin [63], fibronectin [65] have been shown to regulate cell cycle progression [66], functional differentiation [67], and regulation of apoptosis [68]. In the absence of such crucial regulatory proteins, loss of differentiation occurs as well as increased apoptosis [66, 69, 70].

20.2.4 The BM Influences Retinal Blood Flow

Retinal blood flow is regulated, at least in part, by pericyte contractility and relaxation [71]. When pericytes relax, their processes spiraling the retinal microvessels allow an increase in vessel caliber. Inversely, when the pericytes contract, retinal microvessel diameter is reduced. Through these relaxation and contractility modes of action, pericytes, at least in part, regulate retinal blood flow. Pericytes, along with the endothelial cells in the retinal microvessels, contribute to the synthesis of BM components and their loss could result in hyperdilation of the microvessels that could impact retinal blood flow [71]. Furthermore, studies have indicated that under hyperglycemic condition, BM can stiffen. This, in turn, could affect the elasticity of the vessels, thereby compromising the ability of the pericytes to regulate retinal blood flow [57]. The contractile nature of the pericytes could be compromised under hyperglycemic condition, which could also affect retinal blood flow [72, 73].

20.2.5 The BM Regulates Neovascularization

The ECM regulates neovascularization by carefully regulating molecular mechanisms underlying the activation, proliferation, migration, and survival of endothelial cells. This is then followed by the degradation of BM structures, resulting in the sprouting and progression of new vessel formation. The association of MMPs helps in remodeling of the ECM and also creates an environment for the endothelial cells and pericytes in the synthesis and assembly of BM components. Cytokine signaling and endothelial proliferation during angiogenesis are regulated by the ECM, which harbors and provides the cytokines necessary for angiogenesis [74].

20.3 Regulation of Cell Signaling via Integrin and Cell–Matrix Interactions

The response of a cell to mechanical and biochemical cues of the ECM is conveyed by the integrins and the actin cytoskeleton. The interaction of integrins to the ECM is very specific, as they determine the downstream signaling process and dictate cell behavior and fate. Integrins recognize specific motifs, such as the Arg-Gly-Asp (RGD) domain of the ECM components, which also serve as an attachment site mediated by integrins [75].

One of the main functions of the ECM and the integrins is the mediation of cell surface receptor signaling. ECM serves as a reservoir where growth factors, such as the fibroblast growth factors (FGFs) and vascular endothelial growth factors (VEGFs), are localized and are presented to the cell surface receptors, thereby regulating cell proliferation, migration, differentiation, and apoptosis [60, 75–78].

20.4 How Does Thickened Vascular BM Develop in Diabetic Retinas?

20.4.1 Overexpression of BM Components

Increased expression of the BM components enhances the thickening of the BM. In an hyperglycemic environment such as DR, pericytes and

endothelial cells [18, 79] synthesize increased amount of BM components such as type IV collagen [80–82], laminin [83], and FN [82]. In parallel, there is decreased degradation of the BM components, contributing to an accumulation of BM components, leading to eventual BM thickening. Substantial evidence shown in galactose-fed rats [84], and in a primate model of DR [85], demonstrate that increased synthesis of BM components contributes to BM thickening in DR.

20.4.2 Effect of Polyol Pathway

Apart from other physiological effects, which promote BM thickening, polyol pathway has been intensively studied [84, 86]. In high glucose condition, aldose reductase converts glucose to sorbitol, which is further converted to fructose by sorbitol dehydrogenase [87]. On the other hand, fructose is converted to fructose-3-phosphate and deoxyglucosone, which form advanced glycation end (AGE) products, which mediate the development and progression of DR [88]. Vascular defects in diabetes have been explained through BM thickening in retinas of 6-month diabetic rats [89] and of galactose-fed rats [90]. Hyperglycemia-induced activation of the polyol pathway also has been shown to induce apoptosis of retinal pericytes and endothelial cells resulting in acellular capillaries, an important event in the pathogenesis of diabetic retinopathy [91]. While the effect of polyol pathway extends to other cellular events such as apoptosis in DR, clinical trials using aldose reductase inhibitors did not produce beneficial effects [92].

20.4.3 Activation of Protein Kinase C

Activation of protein kinase C (PKC) is associated with alterations in BM thickening, ECM expansion, vascular permeability, cell growth, and angiogenesis [93]. The activation of PKC induces the expression of type IV collagen, fibronectin, and laminin. Transforming growth factor 1 (TGF-1) and connective tissue growth factor (CTGF) have been shown to mediate BM thickening in diabetes as they regulate ECM accumulation by increasing the production of type IV collagen, fibronectin, and laminin [94]. While inhibitors of PKC successfully reduced BM thickening, indicating a translational benefit for DR [95], however, in clinical trials, such beneficial effects were not found [96].

20.4.4 Advanced Glycation End Products

The exact mechanism by which AGE products promote DR is not well understood. However, in vitro and in vivo studies have shown that elevated level of AGEs is present in retinal vascular cells under hyperglycaemic milieu [97–102]. Such increased levels of AGE products in endothelial cells may interfere with cell survival pathways (ERK, AKT, MAP kinase, PKC) and can impact BM thickening in DR [103–107]. In the context of proliferative DR (PDR), AGE may increase VEGF levels and promote hypoxia via activation of HIF-1 α and ERK pathways [108, 109]. Accumulation of AGE products in pericytes can contribute to pericyte apoptosis, leading to pericyte loss in diabetic retinas [107]. In addition, a study has identified AGE products in the thickened vascular BM in retinas of diabetic rats [110]. Treatment with an AGE inhibitor, pyridoxamine, is known to decrease laminin overexpression and thereby contribute to the reduction in BM thickness [111]. This suggests the possibility that AGEs are linked to BM thickening in DR [112].

20.4.5 Role of BM Thickening in Disease Pathology

Alterations in the BM can impact the severity and progression of microvascular complications of diabetes, such as diabetic nephropathy [113, 114], diabetic neuropathy [115], and DR. Nearly a century ago, Henry Wagener and Russel Wilder from Mayo Clinic reported that DR is nearly always associated with vascular complications of the retina [116]. Nearly five decades later dur-

ing which period, electron microscopy came into vogue, it was confirmed that the retinal vascular complications and vascular BM thickening were closely associated with the development and progression of DR [117]. Clear evidence of increase in BM-related alteration was initially reported in muscles [118] followed by reports confirming that BM thickening is associated with other complications of diabetes such as retinopathy from evidence of capillary BM thickening in STZ-administered diabetic rats [119]. BM thickening has been shown in a variety of tissues and in the natural aging process and is also strongly associated with disorders such as diabetes and hypertension [120]. A recent understanding of the literature suggests that BM thickening is closely associated with the development and progression of DR [11]. However, the consequences of BM thickening are only beginning to be understood. The impact of BM thickening to the disease process of DR can be explained by changes in cellular processes, such as accelerated apoptosis [121] and excess vascular permeability [122]. Attenuation of the overexpression of BM components, such as fibronectin, using antisense oligonucleotides has shown to reduce diabetes-induced retinal vascular permeability in diabetic rats [123].

Among the various complications of diabetes mellitus, microvascular complications that affect the blood vessels are a serious health burden including DR. The effect of hyperglycemia on BM thickness has been documented in many organs (Table 20.1) [6, 124–131].

BM thickening in DR is one of the hallmarks of the disease pathology that has gained more importance due to its role in various functions (Fig. 20.1), such as selective substratum attachment, selective permeability, neovascularization, cell growth, cell proliferation, apoptosis, and signaling. Increased synthesis and accumulation of BM proteins is the major contributor of BM thickening. However, decreased degradation of BM by MMPs in hyperglycemia can also contribute to the overall process of BM thickening.

Evidence from the literature suggest that high glucose exposure alone is sufficient to disrupt the vascular microenvironment homeostasis, resulting in abnormal ECM deposition, ECM assembly, and lead to increased permeability [132], thereby destabilizing the blood–retinal barrier [133]. On the other hand, tight glycemic control has shown to reduce or prevent BM thickening by regulating the synthesis of certain BM components, such as fibronectin, in retinal capillaries [134].

In diabetes, hyperglycemia induces BM thickening, which in turn, contributes to the

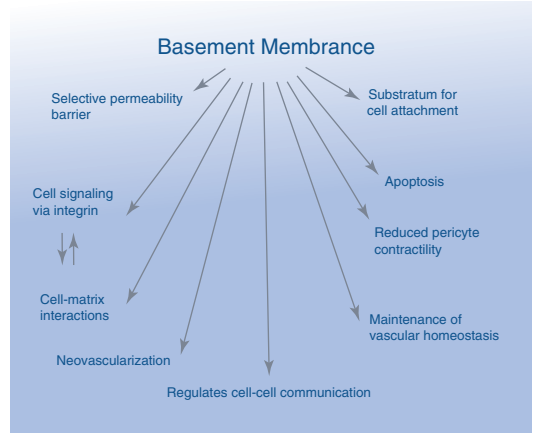

Fig. 20.1 Functions of vascular basement membrane. Vascular BM is a multifunctional unit providing structural support for attachment of endothelial cells and pericytes while participating in the regulation of cellular events including vascular permeability characteristics, apoptosis, vascular homeostasis, cell–cell communication, neovascularization, and cell–matrix interactions

Table 20.1 Vascular basement membrane thickness in different human tissues

Tissue	BM thickness (nM)	References	BM thickness in diabetes (nM)	References
Retina	292 ± 24	[6]	583.1 ± 38.52	[6]
Glomerulus	321 ± 21	[124]	482 ± 151	[125]
Skeletal Muscles	108 ± 2.7	[126]	240.3 ± 11.9	[126]
Quadricep Muscles	118 ± 2.7	[127, 128]	203.1 ± 18.7	[129]
Skin	250 ± 34	[130]	353.3 ± 38	[130]
Lungs	4968 ± 235	[131]	7217 ± 753	[131]

Table 20.2 Retinal capillary basement membrane thickness in different diabetic species

Species	Normal (nM)	Diabetic (nM)	References
Human	292 ± 24	583 ± 39	[6]
Dog	141 ± 25	236 ± 46	[135]
Rat	51	69	[134]
Mouse	93 ± 19	113 ± 9.6	[136]
Porcine	97 ± 11	152 ± 16	[137]
Cat	72 ± 12	114 ± 15	[138]
Marmoset	147 ± 6	244 ± 30	[85]

development of retinal vascular lesions in DR. Diabetes-induced retinal capillary thickness has been documented in several species and an increase in the thickness of the BM has been measured across different species. Table 20.2 shows the influence of diabetes on retinal BM thickness in human [6], dog [135], rat (*Sprague dawley*) [134], mouse (*Mus musculus*) [136], porcine [137], cat [138], and marmoset [85].

Elevated cytokine is a common characteristic of diabetes, and increased expression of TNFα and IL1β has been observed in Müller and glial cells that correlate with the severity of DR [6]. BM thickening is also considered as a natural process of aging and it has been shown that increase in BM thickness is proportional to aging. Age-related changes in BM thickness has been shown previously in seminiferous tubules of the testis, suggesting a 50% increase in BM thickness with age [120]. Capillary BM thickening, pericyte loss, and acellular capillaries have also been observed in the retinas of nondiabetic aged rats [139].

20.5 Treatment and Prevention Measures

Initiatives to reduce BM thickening as a possible treatment strategy against retinal vascular lesions seen in DR have been carried out using various strategies. Treatment using combined antisense oligonucleotides against BM components prevented vascular cell death and reduced capillary leakage in diabetic retinas [123]. Transfection of phosphorothioate antisense oligonucleotides targeting fibronectin effectively reduced high glucose-induced fibronectin overexpression in endothelial cells [140]. Aldose reductase inhibitor (sorbinil) has been shown to effective in reducing vascular BM thickening of the deep capillary bed of retinas [89, 90]. However, the effect of aldose reductase inhibitor as a possible therapeutic agent against BM thickening was found to be ineffective in clinical trials [141]. AGEs, which are considered as a risk factor in BM thickness have also been tested as a therapeutic target in the treatment of DR. Treatment with Pyridoxamine, an inhibitor of AGEs, was shown to reduce laminin mRNA expression in the retinas of diabetic rats and resulted in reduced acellular capillaries [142]. Angiotensin-converting enzyme (ACE) inhibitors have also been shown to be effective in reducing BM thickening in STZ-induced diabetic rats [143]. In a high glucose milieu, the expression of TGF-β is stimulated by increased expression of angiotensin II, resulting in the production of extracellular matrix proteins, such as collagen and fibronectin. ACE inhibitors reverse this effect by inhibiting angiotensin II formation, thereby reducing the production of ECM and increasing its degradation [144–147].

At the present time, there is evidence supporting the notion that BM thickening is not only associated with the development and progression of DR, but also plays a detrimental role during this process. In particular, the abnormal function of thickened vascular BM in the diabetic retina is intrinsically linked to cell death and compromised blood–retinal barrier characteristics. Improved understanding of the pathological role of the thickened BM has triggered intense quest for a better treatment modality to reduce BM thickening and thereby prevent retinal lesions characteristic of DR. Future studies are required for developing efficient interventional strategies using novel drugs for sustained improvement in the fight against DR.

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21 Molecular Genetics and Clinical Aspects of Macular Corneal Dystrophy

Durga Murugan, Sindhura Devi Adsumilli, Namperumalsamy Venkatesh Prajna, and Periasamy Sundaresan

Abstract

The prevalence of macular corneal dystrophy (MCD) varies immensely in different parts of the world. Though MCD is rare corneal dystrophy, consanguinity among the populations increases the risk of occurrence. It is most predominant in Iceland, Saudi Arabia, and South India due to high degree of consanguinity. Unlike in the Western countries, MCD is the most common corneal stromal dystrophy in India. MCD is an inherited, autosomal recessive disorder caused by defective keratan sulfate (KS) metabolism. It is characterized by bilateral, progressive clouding of the corneal stroma with the presence of grayish-white, ill-defined opacities. The clinical manifestations of MCD usually start in the first decade of life leading to progressive visual loss eventually necessitating corneal transplantation by the fifth decade of life. So far, there are limited studies in MCD for understanding the relationship between the mutations in *CHST6* and the mechanism of unsulfated KS deposits. Therefore, understanding the genetic, clinical, and pathophysiological aspects of this complex disease is very essential for the newer treatment options like gene therapies. This chapter provides detailed insight into epidemiology, biochemical mechanism, immunophenotypes, genetics, and clinical aspects of MCD, which enriches our understanding for future research purposes.

Keywords

MCD · CHST6 · Mutation · Keratan sulfate Immunophenotypes

Abbreviations

CHST6	Carbohydrate sulfotransferase 6
KS	Keratan sulfate
MCD	Macular corneal dystrophy

21.1 Introduction

Corneal dystrophies are a group of rare genetically inherited, noninflammatory disorders that are usually bilateral, slowly progressive, symmetric, and not allied with any environmental

D. Murugan · P. Sundaresan (✉)
Department of Genetics, Aravind Medical Research Foundation, Aravind Eye Hospital, Madurai, Tamil Nadu, India
e-mail: sundar@aravind.org

S. D. Adsumilli · N. V. Prajna
Cornea Clinic, Aravind Eye Hospital, Madurai, Tamil Nadu, India
e-mail: prajna@aravind.org

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modification or systemic condition [1]. They are characterized by accumulation of abnormal deposits in the cornea. Most of the dystrophies exhibit an autosomal dominant pattern of inheritance. The most recent iteration of the IC3D guidelines has identified 22 types of corneal dystrophies [2] based on their nature, phenotype, and genotype (Table 21.1). The exceptions to this definition are epithelial basement membrane dystrophy (EBMD) and central cloudy dystrophy of Francois (CCDF) that are considered more likely to be degenerative rather than hereditary conditions. Dystrophies can also be unilateral, as in posterior polymorphous corneal dystrophy (PPCD). Systemic changes such as hypercholesterolemia can be occasionally seen in Schnyder corneal dystrophy (SCD) [2]. Posterior amorphous corneal dystrophy (PACD) is minimally progressive in contrast to other dystrophies [2].

MCD is an autosomal recessive disorder caused by mutations in CHST6 gene [3] that encodes the enzyme carbohydrate sulfotransferase 6, hence can be considered as localized mucopolysaccharidoses [4]. The stromal dystrophies can cause recurrent corneal erosions when superficial, while dystrophies involving deeper layers of the stroma cause more opacification, resulting in decreased vision.

21.2 History of Macular Corneal Dystrophy

In 1890, MCD was first recognized by Groenouw [5] and it was shown to follow an autosomal recessive inheritance pattern [6]. Macular corneal dystrophy is a good example of the historical evolution of knowledge under corneal dystrophy as discussed by Gordon Klintworth. The first corneal graft in MCD was reported by Ernest Fuchs, although he called it Groenouw nodular dystrophy [7]. Oskar Fehr, a German ophthalmologist first differentiated the MCD from granular and lattice dystrophy and hence MCD was also known as Fehr spotted dystrophy [8]. Jones and Zimmerman provide the first evidence defining the histopathologic characteristics and suggested that macular, granular, and lattice dystrophies were distinct entities [9]. Klintworth and Vogel documented the histochemical characterization for MCD in their studies and explained the first transmission electron microscopic analysis for corneal dystrophy [10]. It was first suspected that a glycosaminoglycan was abnormally deposited in MCD due to lack of an enzyme needed for the degradation of keratan sulfate. Later, cell culture studies showed that corneal stromal cells are failed to disclose the evidence of a lysosomal storage disease of keratan sulfate in contrast to the systemic mucopolysaccharidoses [11, 12]. Unlike the normal corneal organ cultures of MCD, corneas failed to produce normal keratan sulfate. Thereafter, other studies showed the discovery of different immunophenotypes (I, IA, II) of MCD, depending on the presence or absence of sulfated keratan sulfate (AgKS) in serum and cornea [12].

21.3 Macular Corneal Dystrophy

Macular corneal dystrophy (MCD; OMIM 217800) is one of the severe forms of IC3D category 1 stromal corneal dystrophy (Fig. 21.1) [13]. Macular corneal dystrophy is an inherited autosomal recessive disorder. It is the most common stromal corneal dystrophy in India as opposed to in Western countries where it is relatively rare [14–17]. It begins in the first decade of life; resulting in progressive visual loss eventually necessitating corneal transplantation [18]. The gene of MCD has been mapped to 16q22 locus of chromosome 16. *CHST6* encodes an enzyme carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6 involved in the sulfation of keratan sulfate (glycosaminoglycan), which plays a role in corneal transparency [3, 19]. Mutations in *CHST6* abolish or reduce the enzyme activity, thus preventing the sulfation of keratan leading to the accumulation of unsulfated keratan (Glycosaminoglycan) in corneal stomal intracellular, extracellular matrix, and keratocytes. This causing increased cloudiness of the cornea leading to decreased visual acuity throughout the life. To date, more than 140 distinct mutations have been identified in *CHST6* and associated with MCD [20].

Table 21.1 Anatomical IC3D classification of corneal dystrophies

S. No.	Affected corneal layers and dystrophies	Category 1 corneal dystrophies (C1)	Category 2 corneal dystrophies (C2)	Category 3 corneal dystrophies (C3)	Category 4 corneal dystrophies (C4)
1.	Epithelial and subepithelial dystrophies	• EBMD (Epithelial basement membrane dystrophy) majority degenerative, rarely—(*TGFβ12)*, AD • MECD (Meesmann corneal dystrophy)—(*KRT3/ KRT1*), AD • GDLD (Gelatinous drop-like corneal dystrophy)—(*TACSTD2*), AR	LECD (Lisch epithelial corneal dystrophy)—(U), XR	EREDs (Epithelial recurrent erosion dystrophies)—FRCD (Franceschetti corneal dystrophy), DS (Dystrophia Smolandiensis) and DH (Dystrophia Helsinglandica)—(U), AD	SMCD (Subepithelial mucinous corneal dystrophy)—(U), AD
2.	Epithelial-stromal TGFβ1 dystrophies	• RBCD (Reis-Bucklers corneal dystrophy)—(*TGFβ1)*, AD • TBCD (Thiel-Behnke corneal dystrophy)—(*TGFβ1*), AD • LCD1 (Lattice corneal dystrophy, type 1)—variants (III, IIIA, I/IIIA, IV) of lattice corneal dystrophy—(*TGFβ1*), AD • GCD1 (Granular corneal dystrophy, type 1)—(*TGFβ1)*, AD • GCD2 (Granular corneal dystrophy, type 2)—(*TGFβ1)*, AD			
3.	Stromal dystrophies	• MCD (Macular corneal dystrophy)—(*CHST6*), AR • SCD (Schnyder corneal dystrophy)—(*UBIAD1*) • CSCD (Congenital stromal corneal dystrophy)—(*DCN*), AD • FCD (Fleck corneal dystrophy)—(*PIKFYVE*), AD • PACD (Posterior amorphous corneal dystrophy)—(*LUM* or *KERA/DCN/ EPYC/CCER1*), AD • PDCD[a] (Pre-Descemet corneal dystrophy) —(*STS*), XR			
4.	Endothelial dystrophies	• FECD[b] (Fuchs endothelial corneal dystrophy)- (*COL8A2/ TCF4*), Sporadic/AD • PPCD[c] (Posterior polymorphous corneal dystrophy)—*COL8A2/TCF4/ZEB1*), AD • CHED (Congenital hereditary endothelial dystrophy)—(*SLC4A11*), AR	XECD (X-linked endothelial corneal dystrophy)—(U), XR		
5.	Removed dystrophies				Grayson-Wilbrandt corneal dystrophy (GWCD) C4

AR, autosomal recessive; AD, autosomal dominant; XR, X-linked recessive; U, unknown gene
[a]PDCD-Under the category of C1, C4
[b]FECD-Under the category of C1, C2, C4
[c]PPCD-Under the category of C1, C2, C4

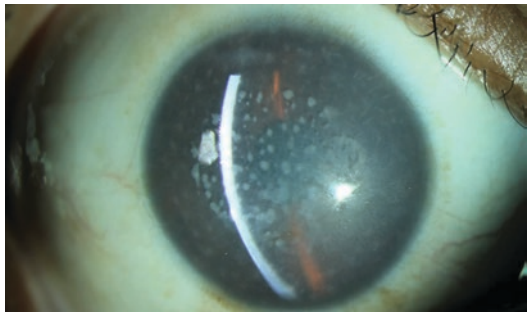

Fig. 21.1 Slit lamp image of cornea with macular corneal dystrophy demonstrating multiple, irregular gray-white opacities with intervening central stromal haze

21.4 Epidemiology and Demographics of MCD

Corneal diseases represent an important cause of blindness and visual impairment after cataract and glaucoma [2]. The epidemiology of corneal blindness is complicated because of the difficulty of treating corneal diseases [21]. The prevalence of corneal diseases varies from country to country. In MCD, a stromal lesion typically starts in the first decade of life and leads to the progressive vision loss by the third decade [2]. MCD has been recognized throughout the world. This wide-ranging frequency of MCD is evidenced by many studies [15–17, 22–27]. The prevalence of MCD varies across the world with the highest incidence in India [15–17], Saudi Arabia [28, 29], and Iceland [23] due to increased rates of mutations in the CHST6 gene. Due to small gene pool in Iceland and high degree of consanguinity in Saudi Arabia and South India, MCD was more predominant in these populations [14, 24, 30–33]. Al Faran et al. reported that 52% of all corneal dystrophies in Saudi Arabia are MCD; with 42% of MCD cases observed in offspring of consanguineous marriages [28]. Furthermore, MCD accounts for a high percentage (10–75%) of corneal dystrophies requiring keratoplasty in Iceland and Japan [23, 27]. In addition, MCD is considered to be the most common among other corneal dystrophies in Asia [34].

In the United States, MCD is a relatively rare disorder with a prevalence of about 0.3 individuals per 2,50,000 inhabitants while in Iceland this number corresponds to 19 individuals. Furthermore, approximately 60% of all corneal dystrophies are endothelial, whereas macular, lattice, and granular corneal dystrophies are far less prevalent, each making up 1% or less of the total [35]. But lattice corneal dystrophy is the most frequent stromal corneal dystrophy than macular and granular corneal dystrophies in this particular population [21]. Moreover, in the United States all the corneal dystrophies were more common among females. And the percentage of females affected by lattice corneal dystrophy (LCD) was higher (68.5%) than macular corneal dystrophy (56.6%) [34].

In 2007, the Australian Corneal Graft Registry reported that 1.9% of MCD cases observed yearly [36]. According to the French National waiting list records, it was only 2% of MCD cases were observed in the French population [37]. Jee et al. reported that MCD accounts for 12.9% of corneal dystrophy encountered in Koreans, following granular dystrophy (29.2%) and Fuchs' endothelial dystrophy (23.6%) [38]. MCD is a rare, least common disorder among other corneal dystrophies in German, Vietnam, Italy, and Iranian populations [39–42].

21.5 Biochemical Mechanism of MCD

Glycosaminoglycans (GAGs) are the major corneal storage material of MCD. MCD is a genetically metabolic disorder of KS catabolism [10, 43, 44]. In MCD, keratan sulfate (KS) is the major proteoglycan (PG), which plays a main role in corneal hydration due to its polar nature of the GAG chain in the presence of sulfate esters. Lumican and keratocan are the major proteoglycans of the corneal stroma that bear keratan sulfate chains [45]. KS is a large complex of negatively charged heteropolysaccharide chain that is composed of a polylactosamine (a repeating N-acetylglucosamine–galactose disaccharide), which is typically sulfated at the 6-O position of N-acetylglucosamine [4, 46]. The unsulfated polylactosamine chains are likely to be less water soluble than the fully sulfated KS due to the decrease in polarity of the GAG chain in the absence of sulfate esters.

Usually, the initiation of sulfation starts with the synthesis of sulfate group donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (Fig. 21.2).

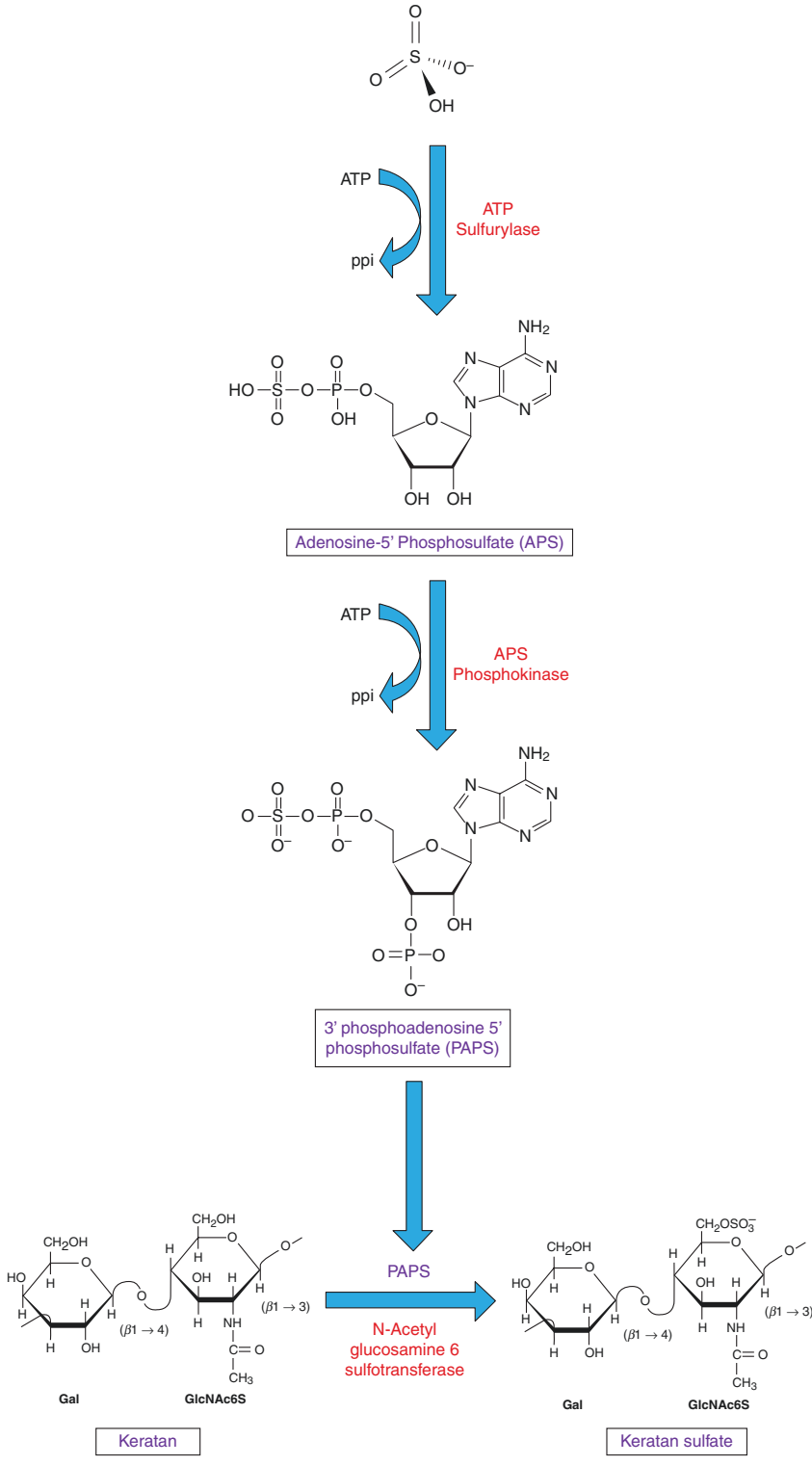

Fig. 21.2 Biochemical mechanism involved in macular corneal dystrophy. Formation of PAPS (sulfate donor) & keratan sulfate: Adenosine-5' Phosphosulfate (APS) is formed with the help of ATP Sulfurylase enzyme. APS is then again phosphorylated at 3' position of ribose sugar by an enzyme APS phosphokinase to ultimately form 3'-phosphoadenosine 5'- phosphosulfate (PAPS). With the impact of sulfate donar PAPS the unsulfated keratan is converted into keratan sulfate for the transparency of cornea

PAPS synthesis is carried out in two steps. In the first step Adenosine-5′ Phosphosulfate (APS) is formed by the catalytic enzyme ATP sulfurylase. ATP sulfurylase catalyzes the activation of sulfate by transferring sulfate to the adenine monophosphate moiety of ATP and forms APS and pyrophosphate (ppi). APS is further phosphorylated at 3′OH of adenosine by an enzyme APS phosphokinase to form PAPS. Finally, N-acetyl-glucosamine-6-sulfotransferase (C-GlcNAc6ST) catalyzes the sulfation of keratan sulfate along with the aid of sulfate donor PAPS. Sulfated keratan sulfate will be maintaining the thickness of collagen fibrils thereby retaining the corneal transparency.

MCD corneas do not synthesize a normal KS (KS I). Because mutations in the CHST6 gene will be immensely affect the 5'PB domain which is an active site of the C-GlcNAc6ST enzyme. This site is responsible for PAPS binding will be abolished by the mutation which leads to the formation of low sulfated or unsulfated KS [12, 47–53]. The unsulfated KS is less water soluble than sulfated KS [50]. The unsulfated KS is unable to be completely metabolized due to loss of its soluble property. Hence the unsulfated KS is deposited in the corneal stromal matrix, keratocyte cells, which lead to corneal opacity resulting in loss of vision [50, 54].

21.6 Clinical Manifestations

Macular corneal dystrophy begins in the first decade of life and results in progressive visual loss as the stroma becomes more cloudier with superimposed dense grayish-white spots with intervening haze. Initially, macular spots are seen in the central cornea in the superficial layers of the stroma but as the age progresses the lesions involve the entire thickness of cornea and approach toward the periphery [18]. Regular astigmatism along with central corneal thinning is characteristic of this dystrophy [55]. Higher-order aberrations are also seen in MCD [56]. Corneal endothelium is primarily involved in MCD resulting in guttae [57].

The stromal dystrophies can cause recurrent corneal erosions when superficial, while dystrophies involving deeper layers of the stroma cause more opacification, resulting in decreased vision/glare/corneal sensitivity [58, 59]. Corneal dystrophies affecting the endothelium are characterized by symptoms of intermittently reduced vision from epithelial/stromal edema; visual acuity worse in the morning because of increased stromal/epithelial edema after overnight eye closure and pain/photophobia/epiphora due to epithelial erosions resulting from ruptured epithelial bullae [60, 61].

The lesions in MCD stain well with alcian blue, colloidal iron, and minimally with PAS and does not stain with Massons trichrome [18]. Histochemically, abnormal keratin sulfate GAGs are seen intracellularly within keratocytes or endothelium and extracellularly in stroma and Descemet's membrane [62]. At cellular level, MCD is characterized by distended cisternae of rough endoplasmic reticulum cisternae and increased lysosomal activity. Accumulated storage products result in engorgement of cells resulting in cell degeneration or rupture. In systemic mucopolysaccharidosis storage products accumulate within intracytoplasmic vacuoles associated with Golgi complex [18].

21.7 Immunophenotypes of MCD

Histochemical and immunohistochemical studies are aimed to differentiate the phenotypes of MCD. Histopathologically, MCD is illustrated by intracellular storage of glycosaminoglycans (GAGs) within keratocytes and corneal endothelium along with an extracellular deposition of similar abnormal material seen in corneal stroma and Descemet's membrane. This abnormal accumulation of KS is positively stained by alcian

blue, periodic acid-Schiff, colloidal iron stains [10, 63, 64]. Histochemical studies showed that these abnormalities are due to error in the glycosaminoglycan metabolism, which results in abnormal deposition of KS [48].

Based on the measurement of antigenic keratan sulfate (AgKS) in serum and the evaluation of AgKS in corneal tissue, MCD can be subdivided into three phenotypes: type I, type IA, and type II (Table 21.2). Immunohistochemically, MCD can be subdivided into three phenotypes (I, IA, and II) based on the serum AgKS levels and the evaluation of AgKS in corneal tissue. The determination of AgKS in blood [65] and tissue [52] uses a monoclonal antibody (1/20/5-D-4), directed against highly sulfated epitopes present in both corneal and skeletal keratan sulfate chains. When keratan sulfate is appropriately sulfated, it reacts with a monoclonal antibody that specifically recognizes sulfated keratan sulfate but when it is not properly sulfated, the antibody does not recognize keratan sulfate [66]. Out of the three immunophenotypes in MCD, type I is the most prevalent one which is characterized by the absence of AgKS in both serum and cornea. Type IA has been marked by the presence of AgKS in corneal stromal keratocytes only but absent in serum [24, 29, 67, 68]. Type II is characterized by the presence of AgKS level in serum but abnormal KS is present intracellularly in keratocytes, endothelium, and extracellularly in stroma and Descemet's membrane. Additionally, Sultana et.al observed an unusual genotype feature in seven MCD families which is named as "atypical" phenotype. Though, it has been shown that immunohistochemical analysis of the corneal sections present with little or no AgKS reactivity along with the serum AgKS levels ranged from 19 to 388 ng/ml [69].

21.8 Genetics of MCD

Developing more scientific approaches for understanding the molecular genetic basis of macular corneal dystrophy is very important for the earlier accurate diagnosis, better treatments, and preventive therapy. In an earlier study by Vance et al. linkage analysis approach was used to identify the genetic locus associated with MCD. This study, for the first time documented the localization of locus for MCD type I mapping to the chromosome 16q21-22.1. This study also suggested the possibility of MCD type II to be in the same locus as for MCD type I [70]. Furthermore, the haplotype analysis of Icelandic families defined the MCD type I gene was localized between the markers D16S3115 and D16S3083 on the long arm of chromosome 16 (16q22) [31]. Later, Hasegawa and co-workers studied the activity of human serum N-acetylglucosamine-6-sulfotransferase (GlcNAc6ST) in normal and MCD patients. In comparison to the normal, this study showed the decreased GlcNAc6ST activity in MCD patients, which could be the possi-

Table 21.2 Classification of MCD immunophenotypes I, IA, II

	AgKS level in MCD type I		AgKS level in MCD type IA		AgKS level in MCD type II	
Country	Cornea	Serum (ng/ml)	Cornea	Serum (ng/ml)	Cornea	Serum (ng/ml)
South India	Absence (or) very low	<2 ± 8	Absence in extracellular stromal matrix, but detected in keratocytes	8	Normal (or) slightly reduced	103–210
America	Absence (or) very low	<2	Absence in extracellular stromal matrix, but detected in keratocytes	2	Normal (or) slightly reduced	282–284
North America	Absence (or) very low	<2–6	Absence in extracellular stromal matrix, but detected in keratocytes	2–6	Normal (or) slightly reduced	207
Egypt	Absence (or) very low	< 10	Absence in extracellular stromal matrix, but detected in keratocytes	10	Normal (or) slightly reduced	112–617
Japan	Absence (or) very low	<0.15	Absence in extracellular stromal matrix, but detected in keratocytes	<0.15	Normal (or) slightly reduced	276

Normal range of sulfated keratan sulfate level in South India: 134–515 ng/ml

ble reason for low or unsulfated KS. This study revealed the importance of GlcNac6ST enzyme that plays a main role in the KS biosynthesis and exhibits a critical role in MCD [71, 72].

Subsequently, Akama and colleagues identified a new carbohydrate sulfotransferase gene (*CHST6*) that encodes an enzyme CGlcNAc6ST within the locus mapped for MCD type I. In situ hybridization and immunohistochemistry was used to analyses the expression profile of *CHST6* gene in human cornea. Further, they also looked for the mutations in the coding regions of *CHST6* by using PCR followed by direct sequencing. They identified four different missense mutations, one frameshift mutation, and a deletion mutation within the coding region of *CHST6* in MCD type I patients. They found two DNA rearrangements in the upstream regions of *CHST6* in the case of type II MCD patients [4]. Consequently, several other studies also reported the involvement of *CHST6* genetic mutations in type I and II MCD patients from different ethnic populations [4, 33, 39–42, 52, 65, 66, 73–76].

Several genetic studies have illustrated the different types of mutations in *CHST6* in different ethnicities have been expanding the mutational spectrum of MCD. Missense mutations in the coding region of *CHST6* have been suggested as a cause for MCD type I. These missense mutations in *CHST6* lead to the functional inactivation or protein degradation or intracellular mislocalization of the enzyme, which is required for the production of sulfated KS [77]. Conversely, MCD type II occurs due to deletions or rearrangements in the region between *CHST5* and *CHST6* genes or in the upstream region of *CHST6* gene. This critical region may include a gene regulatory element that affects the transcription of *CHST6* which leads to the loss of cell-specific *CHST6* expression specifically in corneal cells, but not in other KS rich tissues such as cartilage. This may explain the difference in serum sulfated KS levels between MCD types I and II, although their clinical phenotype is indistinguishable [4]. Furthermore, Niel et al. suggested that frameshift mutations are also expected to be associated with a clinically more severe phenotype [65]. Moreover "atypical" immunophenotype is also identified which mainly occurs due to missense and nonsense mutations in the *CHST6* coding region as well as due to negative mutations (no coding region mutation). This study showed no correlations between *CHST6* mutations and immunophenotypes, therefore suggesting different immunophenotypes which could be explained by the factors other than *CHST6* mutations [69].

In Southern India, several studies reported the different types of mutations in *CHST6*, contributing to the increased mutational landscape for *CHST6* [14–17, 73]. These identified known mutations (except novel mutations) have been observed among the patients from several populations including North India, Saudi Arabia, Korea, Egypt, America, France, South Africa, Chinese, Germany, Italy, Iran, and Vietnam [4, 33, 39–42, 52, 65, 66, 73–76]. This additionally supports the presence of high degree of mutational heterogeneity among the MCD patients. Sultana and co-workers revealed that MCD type I is the most common immunophenotype seen in Indian patients [69]. The prevalence of MCD type I was similar to the studies on various ethnicities including Iceland, Saudi Arabia, and the United States [24–26, 29].

In another study, whole exome sequencing was performed using a consanguineous black South African family to investigate the MCD causal mutations. This study identified a homozygous missense mutation (E71Q) in two affected sisters. This study also predicted that this mutation is probably damaging, disease causing, and deleterious effect on the enzyme (CGlcNAc6ST) using bioinformatics softwares including Polyphen2, MutationTaster2, and SIFT, respectively [74].

In addition, Wang et Al. used ten MCD affected Chinese families for direct Sanger sequencing analysis. They identified 3 novel mutations and 7 previously reported mutations consisting of deletions, insertions, missense, and nonsense mutations. Moreover, they investigated the role of endoplasmic reticulum (ER) stress and apoptosis in keratocytes of MCD patients. This

study suggested that mutations in *CHST6* may trigger ER stress with considerable upregulation of stress marker proteins (GRP78/CHOP) and cell apoptosis [78].

21.9 Imaging

Confocal microscopy shows areas of altered reflectivity in basal epithelial cells and hyper reflective areas in anterior stroma along with granular appearance of stromal keratocytes and extracellular matrix. Dark striae of different lengths and orientations can be found in the middle and posterior stroma. The corneal endothelium may show polymegathism with bright granules in the cytoplasm [79]. Ultrasound biomicroscopy shows deposits in stroma with posterior corneal changes including deep opacities and focal protrusions of the posterior cornea. Anterior segment-OCT shows hyperreflective accumulation in extracellular matrix with opacities in posterior stroma and endothelium. Pentacam Scheimpflug imaging of MCD shows posterior float elevation, thinning at the site of corneal ectasia. Light microscopy shows corneal fibroblasts distended with storage material. Transmission electron microscopy shows abnormal keratocytes distended with membrane-bound vesicles containing fibrillary material, extracellular material interspersed between collagen lamellae, honeycomb vacuoles with interspersed fibrous long-spacing collagen fibers in Descemet's membrane and delicate fibrillary material in corneal endothelium [80–82].

21.10 Treatment

The preferred treatment for MCD has not yet been established. Deep anterior lamellar keratoplasty (DALK) is preferred in MCD patients in whom endothelium is not involved [83]. PKP is the preferred treatment of choice in MCD patients where endothelium is involved [84, 85]. Peripheral clinical recurrence is observed in patients who underwent PKP and the size of the graft used is inversely related to the recurrence in eyes undergoing PKP [86]. However, recurrences are found to occur in patients who underwent DALK because of the left out diseased tissue. Recurrences occur due to subclinical involvement of endothelium, which can only be diagnosed by histology or by ultrasound biomicroscopy (UBM). Recurrences occur at various locations such as subepithelial or at surgical interface. The difference in location is found to be related to the differences in migration of diseased keratocytes and/or GAGs. A study reported more severe phenotypes in patients with frameshift mutations as compared to those with missense mutations [39]. However, another study did not identify any meaningful genotype–phenotype correlations [87]. Hence mutation type by itself cannot serve as a criterion to decide on PKP/DALK. Although MCD has been extensively studied, very little information on phenotype–genotype correlations are available [88]. Recurrence is comparatively infrequent in macular and Schnyder's crystalline dystrophy as compared to lattice dystrophy [89]. Preceding Phototherapeutic Keratectomy (PTK) does not appear to impair the outcome of subsequent penetrating keratoplasty in stromal corneal dystrophy patients [90]. Visual and refractive outcomes are comparable between DALK and PK groups. DALK was superior to penetrating keratoplasty (PKP) in its safety against postoperative complications such as endothelial rejection and secondary glaucoma [91]. In stromal dystrophies, phototherapeutic keratectomy (PTK) was effective in removing large subepithelial stromal plaques. There were no subepithelial recurrences, and hemidesmosome density was increased [92]. In superficial opacities that are caused by macular corneal dystrophy, PTK can increase best corrected visual acuity (BCVA) moderately for a limited period of time [93]. Microkeratome-assisted anterior keratectomy, anterior lamellar keratoplasty, or PTK are suitable therapeutic interventions for superficial and anterior stromal corneal pathology [94]. More recently, femtosecond laser-assisted keratectomy (FLK) has been advocated for MCD [95].

21.11 Recurrence

Recurrence is defined as any clinical findings compatible with recurrence of the disease in the graft button. Clinically significant recurrence is defined as occurring in the visual axis causing decreased visual acuity (loss of two lines or more, or worse than 20/40) or causing recurrent erosion symptoms. Recurrence in macular corneal dystrophies is rare and next to bowman's and Granular corneal dystrophies [96]. One of the causative factors is that endothelial cells of the recipient human cornea may have resurfaced Descemet's membrane of recipient cornea after penetrating keratoplasty. Another alternative possibility is the involvement of both corneal endothelium and Descemet's membrane in macular dystrophy is a secondary phenomenon, and that the abnormal storage product of this dystrophy is an abnormal metabolite rather than a normal compound with a defective catabolic enzyme [81, 84, 97, 98]. Newer techniques such as gene-targeting therapies and enzyme replacement therapies are being studied for a potentially permanent solution in macular corneal dystrophy. Recent research is directed toward development of genetically modified products to integrate into host corneal DNA and block the mutant genes and hence overcome the underlying pathophysiology [82].

21.12 Newer Treatment Options

Although the candidate gene for MCD has been identified and newer promising modalities of gene therapies such as genetic editing, siRNA have recently been available, gene therapy to cure MCD has not yet been reported in any human patient [99]. Furthermore, there are very few circumstances under which gene therapy can be considered as an alternative to keratoplasty in MCD. Gene editing for corneal dystrophies using CRISPR/Cas9 tools have advantages and disadvantages [100–103]. For autosomal recessive disorders Non-homologous end joining (NHEJ) may be used to inactivate the mutated allele. Successful use of CRISPR/Cas9 in Burkitt's lymphoma and hereditary tyrosinemia has been established [104, 105]. An allele-specific siRNA has been shown to completely block mutant keratin12 protein expression in limbal epithelial stem cells grown from patients with Meesmann epithelial corneal dystrophy (MECD) [106]. Gene encoding for SLC4A11 protein is mutated in Congenital Hereditary Endothelial Dystrophy (CHED). Newer treatments to relocate the mutant protein from endoplasmic protein to plasma membrane have to be developed to treat CHED. In vitro model for Gelatinous drop-like corneal dystrophy (GDLD) of immortalized epithelial cell line lacking functional tumor calcium signal transducer 2 (TACSTD2) [107]. A siRNA specific for TGFB1-Arg124Cys to silence the expression of TGFB1 protein has been developed in in vitro animal models of lattice corneal dystrophy type 1 [108–110]. Due to relative paucity of animal models for macular corneal dystrophies and multiple mutations involved in MCD, gene therapy for MCD has to overcome many challenges before its therapeutic use in humans can be demonstrated. Identification of a specific mutation for a given individual is needed and a personalized approach is necessary in view of multiple mutations identified in MCD and also due to poor geno–phenotypic correlation [82, 103].

21.13 Conclusion

Although recently multiple surgical treatment options such as PTK, DALK, and PKP have been available for MCD; gene therapy remains a challenge due to paucity of in vitro models, difficulty in identifying multiple genetic mutations, high degree of allelic and locus heterogeneity, poor geno–phenotypic correlation, and also due to complex understanding related to pathophysiology of distribution of keratan sulfate. It can well be stated that it is too early to comment on application of gene therapy for MCD. Further research is critically important to resolve these unanswered questions related to MCD.

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Congenital and Inherited Cataracts

22

J. Fielding Hejtmancik, Sohan Rao, Maria Lee, Celestine Zhao, William Fielding, Luke Xia, Elizabeth Fielding, Manuel Datiles III, and Alan Shiels

Abstract

Congenital cataracts cause approximately one-third of blindness in infants worldwide. If untreated they can cause permanent blindness by interfering with the sharp focus of light onto the retina and thus fail to establish appropriate synaptic connections between the retina and the visual cortex. Between 8 and 30% (see later) of congenital cataracts are inherited, and our understanding of their genetic architecture is increasing. Delineating the relationship between the genes and mutations causing cataracts and their phenotypic presentation can help us to understand the biology of the lens and provide a framework for the clinical approach to diagnosis and treatment.

Keywords

Cataract · Lens · Congenital · Genetic

J. F. Hejtmancik (✉) · S. Rao · M. Lee · C. Zhao
W. Fielding · L. Xia · E. Fielding · M. Datiles III
Ophthalmic Genetics and Visual Function Branch, National Eye Institute, National Institutes of Health, Bethesda, MD, USA
e-mail: hejtmancikj@nei.nih.gov; datilesm@nei.nih.gov

A. Shiels
Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, MO, USA
e-mail: shiels@wustl.edu

22.1 Introduction

The main functions of the lens are to transmit and focus light onto the retina. The lens transmits light with wavelengths from 390 to 1200 nm efficiently, extending above the limit of visual perception (about 720 nm). Lens transparency results from appropriate architecture of lens cells and tight packing of their proteins, resulting in a constant refractive index over distances approximating the wavelength of light [1, 2]. There is a gradual increase in the refractive index of the human lens from the cortex (1.38) to the nucleus (1.41) where there is an enrichment of tightly packed γ-crystallins.

Cataracts have multiple causes, but are often associated with breakdown of the lens microarchitecture [3, 4], possibly including vacuole formation, which can cause large fluctuations in density resulting in light scattering. In addition, light scattering and opacity will occur if there is a significant amount of high molecular weight protein aggregates 1000 Å or more in size [5, 6]. The short-range ordered packing of the crystallins is important in this regard. For transparency, crystallins must exist in a homogeneous phase.

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22.2 Epidemiology and Global Perspective

Hereditary cataracts are estimated to account for between 8.3 and 30 (see later) percent of congenital cataracts, depending on the population and study [7–9]. For the most part, these differences relate to the higher frequencies of environmental and infectious etiologies in developing nations, lowering the fraction of inherited cataracts, with underlying mutation rates relatively constant. Frequencies of inheritance patterns also relate to marriage patterns in specific populations. For example, about 85% of reported inherited cataracts worldwide are autosomal dominant (see below), while in Pakistan, which has a high rate of consanguineous marriages, about 87% of genetic cataracts are inherited as an autosomal recessive trait [10]. Similarly, it has been estimated that 71% of inherited congenital cataracts in Saudi Arabia are autosomal recessive [11].

22.3 Etiology

In contrast to age-related cataracts, which have a strong environmental component, hereditary congenital cataracts are almost completely determined by germline mutations, which may present as autosomal dominant, autosomal recessive, or X-linked traits. Clinically identical cataracts can result from different mutations and even involving different genes and be inherited in different patterns. Conversely, morphologically distinct and variable cataracts can result from a single mutant gene in a single large family [12]. The number of known cataract loci has increased dramatically in the last few years to well over 60 loci at which mutations in over 40 genes have been demonstrated to cause inherited human cataracts, with the best indications being that approximately 40% of cataract loci have been identified. Obviously, much remains to be learned about the genetic contributions to inherited congenital cataracts.

The genetic architecture of Mendelian cataracts largely comprises a limited number of functional groups making up biological pathways or processes critical for lens development, homeostasis, and transparency (Table 22.1). About a third of cataracts result from mutations in lens crystallins; about a quarter result from mutations in transcription or growth factors; slightly less than one-seventh result from mutations in connexins, about one-tenth result from mutations in membrane proteins or components, and somewhat less than 5% show mutations in chaperone or protein degradation components each, about 2% result from mutations in a mixed group of other genes while the genes at about 3% of known cataract loci have not been identified yet (Fig. 22.1). A more complete list with detailed descriptions and references can be found in CATMAP [13].

The lens has a single layer of anterior epithelial cells overlaying the fiber cells wrapped onion-like around the lens nucleus [14]. Cell division occurs in the germinative zone just anterior to the equator, and the cells then move laterally toward the equator, where the anterior epithelial cells begin to elongate and form secondary fibers [15]. The organelle-rich anterior epithelial cells are connected by gap junctions [16], which facilitate exchange of ions and other low molecular weight metabolites, but tend to lack tight junctions, which would seal the extracellular spaces to these molecules [17]. Differentiating lens fiber cells move toward the lens core and lose their organelles, including the cell nuclei, mitochondria, Golgi bodies, and both rough and smooth ER. Fiber cells, have many interdigitations with minimal extracellular space [18] and are joined by frequent junctional complexes allowing for intercellular transfer of metabolites [19]. Both the anterior epithelial cells and especially the fiber cells contain large amounts of crystallins, as well as cytoskeletal proteins. The process of lens differentiation with its changing protein components are largely under transcriptional control.

Table 22.1 Loci, genes, and phenotypes for nonsyndromic cataract

Gene	Inheritance	Associated extralenticular phenotypes	MIM no.	Gene/locus MIM no.	Locus
1. Transcription and developmental factors					
PITX3	AD	Anterior segment mesenchymal dysgenesis, microphthalmia, neurodevelopmental abnormalities	610623	602669	10q24.32
EPHA2	AD/AR	Susceptibility to age-related cortical cataract	116600	176946	1p36.13
HSF4	AD/AR		116800	602438	16q21
MAF	AD	With or without microcornea	610202	177075	16q22-q23
SIPA1L3	AR		616851	616655	19q13.1-13.2
NHS	X-linked	Nance-Horan (cataract dental) syndrome	302200	300457	Xp22.13
2. Lens crystallins					
CRYGB	AD		615188	123670	2q34
CRYBA2	AD		115900	600836	2q34
CRYGC	AD	With or without microcornea	604307	123680	2q33.3
CRYGD	AD	With or without microcornea	115700	123690	2q33.3
CRYGS	AD		116100	123730	3q27.3
CRYAB	AD/AR	Myopathy, multiple types	613763	123590	11q22.3
CRYBA1	AD		600881	123610	17q11.2
CRYAA	AD/AR	With or without microcornea, susceptibility to age-related nuclear cataract	604219	123580	21q22.3
CRYBB2	AD	With or without microcornea	601547	123620	22q11.23
CRYBB3	AD/AR		609741	123630	22q11.23
CRYBB1	AD/AR		611544	6009291	22q12.1
CRYBA4	AD		610425	123631	22q12.1
3. Gap junction proteins (Connexins)					
GJA8	AD/AR	With or without microcornea	116200	600897	1q21.1
GJA3	AD		601885	121015	13q12.1
4. Membranes and their proteins					
WFS1	AD	Wolfram syndrome (DIDMOAD)	116400	606201	4p16.1
LEMD2	AR		212500	616312	6p21.31
AGK	AR	Senger's syndrome	614691	610345	7q34
MIP	AD		615274	154050	12q13.3
LIM2	AR		615277	154045	19q13.41
LSS	AR		616509	600909	21q22.3
5. Besded filament and other intermediate filament proteins					
BFSP2	AD	Myopia	611597	603212	3q22.1
VIM	AD		116300	193060	10p13
BFSP1	AR		611391	603307	20p12.1
6. Chaperones and protein degradation					
FYCO1	AR		610019	607182	3p21.31
UNC45B	AD		616279	611220	17q12
CHMP4B	AD		605387	610897	20q11.21
7. Other genes and pathways					
TDRD7	AR		613887	611258	9q22.33
GCNT2	AR	Adult i blood group phenotype	110800	600429	6p24
8. Unknown loci					
?	AD		115665	NA	1pter-p36.13
?	AR	With or without microcornea	612968	NA	1p34.3-p32.2
?	AD		115800	NA	2pter-p24

(continued)

Table 22.1 (continued)

Gene	Inheritance	Associated extralenticular phenotypes	MIM no.	Gene/locus MIM no.	Locus
?	AD		607304	NA	2p12
?	?	Susceptibility to age-related cortical cataract	609026	NA	6p12-q12
?	AR		605749	NA	9q13-q22
?	AD		614422	NA	12q24.2-q24.3
?	AD		115650%	NA	14q22-q23
?	AD		605728	NA	15q21-q22
?	AD		601202	NA	17p13
?	AD		115660	NA	17q24
?	AR		609376	NA	19q13

Further information and references can be found at CAT-MAP: https://cat-map.wustl.edu/ [13]

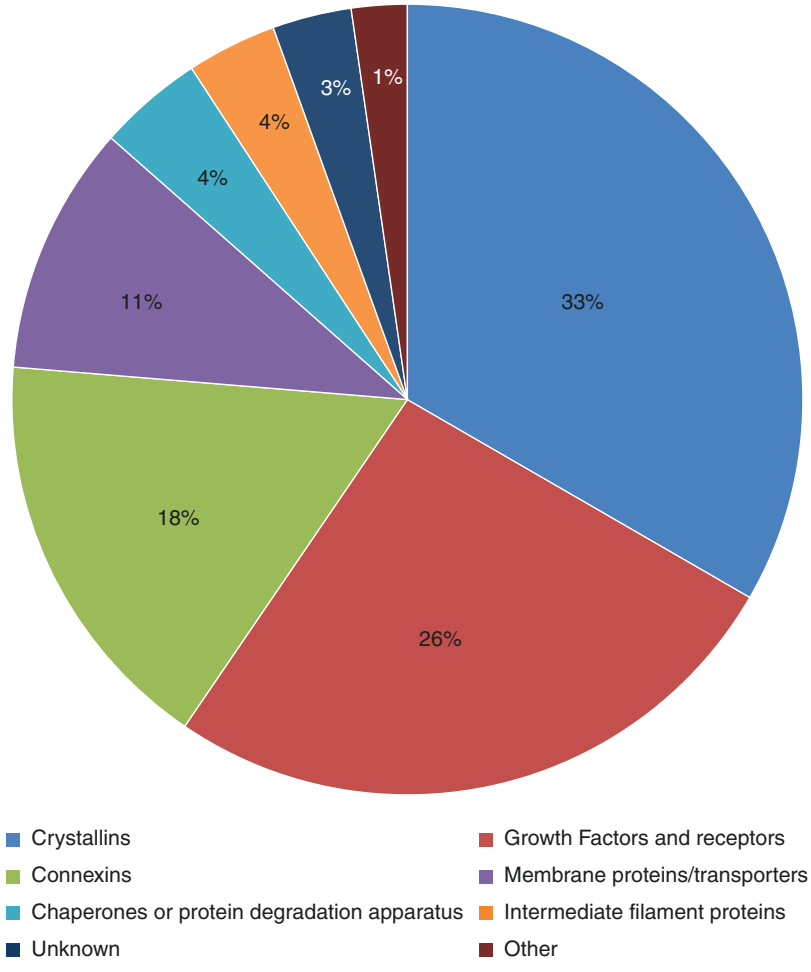

Fig. 22.1 Fraction of cataract families with mutations in genes belonging to specific pathways, processes, or protein families. Crystallins are the most commonly mutated genes in congenital cataract, followed closely by growth factors, connexins, and then membrane proteins. The remainder is caused by additional groups of genes important in a variety of metabolic and functional processes in the lens

22.4 Transcription and Developmental Factors

Although the process and mechanisms of lens development are still being elucidated, a number of transcription and developmental factors including *Pax6*, Rx, VSX2, MAF, FOXE3, EYA1, and PITX3 are critical for lens development [20–25]. Mutations in Pax6, which is expressed in the entire developing eye field, often are associated with aniridia, which is often accompanied by cataracts [26]. Mutations in PITX3 often cause posterior polar cataracts (70%), often associated with anterior segment mesenchymal dysgenesis (ASMD or ASD, affecting the lens, cornea, and iris). Mutations in NHS most often cause the Nance Horan syndrome (NHS), which includes cataracts, facial dysmorphism, dental abnormalities, and often developmental delay and mental retardation. Mutations in NHS often cause nuclear (39%) or sutural (39%) cataracts. In contrast, although it is expressed across most ocular tissues, mutations in HSF4 (heat shock factor 4) tend to cause isolated nuclear or lamellar cataracts as do mutations in *SIPAIL3*, which functions in epithelial cell morphogenesis and polarity. Overall, most mutations in transcription and developmental factors tend to result in autosomal dominant cataracts with a ratio of about 2.5/1, an interesting exception being MAF, which shows no autosomal recessive inheritance in ten independent families identified. Mutations in *TDRD7*, a widely expressed Tudor domain RNA binding protein of RNA granules that interact with STAU-1 ribonucleoproteins also cause cataract, probably related to the high levels of mRNA synthesis required during lens differentiation. Also included in this group is the ephrin receptor EPHA2, which, while not actually a transcription factor, but plays a major role in developmental processes in the eye and nervous system. Mutations in EPHA2 can cause both dominant and recessive congenital cataracts, as well as contributing to age-related cataracts [27–32].

22.5 Lens Crystallins

Crystallins are the most highly expressed proteins in the lens, comprising about 90% of the soluble protein. Their physical properties, especially close packing and stability, are critical for lens transparency. Both of these characteristics are probably responsible for the crystallins being the most commonly mutated genes implicated in human congenital cataracts. There are three classes of crystallins in humans encoded by multiple genes. The β-, and γ-crystallins are part of a large gene superfamily including spore coat protreins. The α-crystallins, comprising αA- and αB-crystallins, part of the small heat shock protein family, have chaperone-like activity binding but not recycling partially denatured proteins and forming large protein complexes with a protective role in the lens. In contrast to αA-crystallinα, which is largely confined to the lens, αB-crystallin is is found in multiple other tissues as well, binding but not recycling partially denatured proteins.

As damaged or mutant β- and γ-crystallins start to form irreversible aggregates that would eventually precipitate out of solution, they are bound by α-crystallins and held in soluble aggregates. However, if the mutation is severe enough to result in rapid denaturation without an intermediate molten globule state, they can escape binding by the α-crystallins and other chaperones in the lens, causing direct damage to the lens cells or initiating cellular processes such as the unfolded protein response (UPR) and apoptosis [33]. Similarly, although most pertinent to age-related cataracts, denaturation and binding of large amounts of crystallins can lead to high molecular weight aggregates large enough to scatter light themselves, and eventually overwhelm the α-crystallin chaperone system causing cataract [34]. Thus, denatured crystallins can lead to cataract directly by scattering light or more catastrophically by toxic effects on the lens cells and micro-architecture perhaps inducing the UPR and/or apoptosis [35].

As would be expected from the discussion above, most cataracts resulting from mutations in crystallins are autosomal dominant, with a

ratio of about 12:1 dominant to recessive. They are heavily biased toward nuclear or lamellar cataracts except for CRYAB cataracts of which 40% are posterior polar and CRYBB3 cataracts, 50% of which are cortical (Table 22.2). This is consistent with most crystallin mutations causing cataract by the proteins gaining a deleterious function, e.g., denaturing and precipitating with a toxic effect on the lens cell, thus inducing the UPR. There is growing support for this mechanism for a variety of crystallin and other mutations [36–40], although some crystallin mutations cause autosomal recessive cataracts. These include CRYAA (3 of 41), CRYAB (5 of 16), CRYBB1 (6 of 19), and CRYBA4 (1 of 5), suggesting that these crystallins might have additional functions in the lens than that of a structural crystallin. The α-crystallins are well known to function as molecular chaperones, but additional functions for the β-crystallins remain to be identified, and no recessive mutations have been identified for any γ-crystallin. Alternatively, mere haploinsufficiency for the crystallins causing autosomal recessive cataracts might be sufficient to impair lens transparency and function.

22.6 Gap Junction Proteins (Connexins)

Lacking blood vessels, the lens is dependent on gap junctions, intercellular channels composed of hexameric hemichannels from two adjacent cells joined to create gap junction channels, for communication and transfer of nutrients, especially between fiber cells. Lens junctions con-

Table 22.2 Clinician characteristics of cataracts by their genetic cause. (**a**) Fraction of mutations in specific genes resulting in cataracts of various morphologies. (**b**) Inheritance patterns for cataracts caused by specific genes

a

	GJA8	GJA3	CRYAA	CRYAB	CRYBB1	CRYBB2	CRYBB3	CRYBA3	CRYBA4	CRYGC	CRYGD
nuclear	0.56	0.51	0.59	0.40	0.75	0.33	0.50	0.47	0.50	0.76	0.43
lamellar [a]	0.26	0.29	0.22	0.20	0.00	0.17	0.00	0.22	0.33	0.24	0.07
sutural	0.06	0.05	0.02	0.00	0.06	0.04	0.00	0.19	0.00	0.00	0.02
cortical	0.00	0.02	0.02	0.00	0.13	0.13	0.50	0.08	0.00	0.00	0.02
PP[b]	0.06	0.05	0.05	0.40	0.06	0.00	0.00	0.03	0.00	0.00	0.05
AP[c]	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.02
corralliform	0.00	0.07	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.32
cerulean	0.00	0.00	0.00	0.00	0.00	0.25	0.00	0.00	0.00	0.00	0.07
PSC	0.06	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00
% defined[d]	0.58	0.77	0.89	0.56	0.55	0.65	0.50	0.91	0.75	0.62	0.79
% other[e]	0.42	0.23	0.11	0.44	0.45	0.35	0.50	0.00	0.25	0.38	0.21

CRYGS	NHS	HSF4	EPHA2	FOXE3	MAF	PITX3	BFSP1	BFSP2	AQP0	GCNT2	FYCO1
0.11	0.39	0.25	0.50	0.25	0.33	0.06	0.50	0.17	0.45	0.50	1.00
0.33	0.00	0.45	0.05	0.00	0.25	0.00	0.25	0.17	0.15	0.25	0.00
0.22	0.39	0.05	0.00	0.00	0.00	0.00	0.00	0.42	0.15	0.00	0.00
0.33	0.18	0.20	0.27	0.38	0.00	0.06	0.25	0.25	0.15	0.00	0.00
0.00	0.00	0.00	0.09	0.00	0.25	0.71	0.00	0.00	0.05	0.00	0.00
0.00	0.00	0.05	0.05	0.00	0.08	0.00	0.00	0.00	0.00	0.25	0.00
0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.00	0.00	0.00	0.00	0.38	0.08	0.00	0.00	0.00	0.05	0.00	0.00
0.00	0.00	0.00	0.05	0.00	0.00	0.18	0.00	0.00	0.00	0.00	0.00
1.13	0.50	0.71	0.96	0.42	0.63	0.61	0.57	1.00	0.61	0.27	0.67
0.00	0.50	0.29	0.04	0.58	0.37	0.39	0.43	0.00	0.39	0.73	0.33

Table 22.2 (continued)

b

	GJA8	GJA3	CRYAA	CRYAB	CRYBB1	CRYBB2	CRYBB3	CRYBA3	CRYBA4	CRYGC	CRYGD
AD	49	44	38	11	13	28	3	26	4	30	51
AR	4	1	3	5	6	0	2	0	1	0	0
AD/AR	12.25	44.00	12.67	2.20	2.17	na	1.50	na	4.00	na	na
group ratio	18.60		12.47								
% AD	0.92	0.98	0.93	0.69	0.68	1.00	0.60	1.00	0.80	1.00	1.00

CRYGS	NHS	HSF4	EPHA2	FOXE3	MAF	PITX3	BFSP1	BFSP2	AQP0	GCNT2	FYCO1	Total
8	0	16	18	6	10	27	2	8	29	0	0	421
0	0	7	5	11	0	1	2	2	1	12	14	77
na	na	2.29	3.60	0.55	na	27.00	1.00	4.00	29.00	0.00	0.00	5.47
			2.54				2.50		varied			
1.00	na	0.70	0.78	0.35	1.00	0.96	0.50	0.80	0.97	0.00	0.00	0.85

[a]Lamellar or zonular
[b]Posterior polar
[c]Anterior polar
[d]Described as one of the above morphologies
[e]Not described or other morphology

tain *GJA3* (encoding connexin 46) and *GJA8* (encoding connexin 50) [41, 42]. Mutations in *GJA3* and *GJA8* have been implicated largely in autosomal dominant human cataract (92% and 98%, respectively) with a few autosomal recessive families reported for each. They also usually cause nuclear or lamellar cataracts (Table 22.2). Because of their multimeric nature, some missense mutations in connexins can have a dominant negative effect on gap junction function as exemplified by the p.P88S change in GJA8, [43]. The mutant protein is incorporated into the gap junction structure and inactivates the entire junction [44]. Other connexin mutations do not inhibit channel function by normal connexins synthesized from unaffected genes but might be retained in or near the endoplasmic reticulum such as the p.46fs380 change or fail to be incorporated into the gap junction at all [45] such as a p.N63S missense mutation, both in GJA3. Some gap junction mutations causing retention in the endoplasmic reticulum can induce the UPR [46], and conversely, mutations causing enhanced hemichannel function also can lead to cell death and cataract [47]. GJA8 mutant cataracts have also been associated with microcornea with or without myopia and occasionally with microphthalmia while GJA3 mutations are usually isolated.

22.7 Membranes and Their Proteins

In addition to the Gap Junction Proteins, lens epithelia require large amounts of membranes when they elongate to form fiber cells and must synthesize the lipids making up the membranes as well as the protein components required for circulation of water and small molecules critical for lens fiber cell homeostasis and function. Mutations in *SLC16A12*, a transmembrane protein functioning in creatine transport can cause dominant cataracts, sometimes accompanied by microcornea or renal glycosuria. Aquaporins are integral membrane proteins that generally act as water channels. Mutations in aquaporin 0 (*AQP0*, also known as major intrinsic protein, *MIP*) are also a major contributor to inherited congenital cataracts, usually nuclear, with some lamellar, sutural, or cortical (Table 22.2). Similar to some gap junction mutations, autosomal dominant

E134G and T138R mutations inhibit normal trafficking of AQP0 to the plasma membrane [48] and also interfere with water channel activity by normal AQP0, consistent with a dominant negative mechanism. *LIM2* is required for cell junctions in lens fiber cells.

TMEM114, a transmembrane glycoprotein member of a group of calcium channel gamma subunits, can also cause cataracts when mutated. Mutations in LEMD2, a transmembrane protein found in the nuclear membrane important for nuclear organization and cell signaling, can also cause autosomal recessive cataracts. While mutations in the wolframin ER transmembrane glycoprotein (WFS1) usually cause Wolfram syndrome, they have also been described in a family with isolated cataracts. Mutations in acylglycerol kinase (*AGK*), a mitochondrial membrane protein, acts as a lipid kinase required for synthesis of phosphatidic and lysophosphatidic acids are associated with autosomal recessive cataracts, as are mutations in lanosterol synthase (LSS), which is required for synthesis of cholesterol. These are possibly related to the large amounts of membrane components required to be synthesized during fiber cell differentiation, although lanosterol has been shown to act as a chaperone for denatured crystallins [49].

22.8 Beaded Filament and Other Intermediate Filament Proteins

Intermediate filaments are cytoskeletal proteins with an average diameter of around 10 nm. In the lens, these include vimentin filaments, which are present in the anterior epithelial cells but are replaced by lens-specific beaded filaments as the cells differentiate into fiber cells. Beaded filaments are composed of BFSP1 (CP115, filensin) and BFSP2 (CP49, phakinin), both highly divergent members of the intermediate filament protein family. About 50% of mutations in BFSP1 cause nuclear cataracts while about 42% of mutations in BFSP2 cause sutural cataracts (Table 22.2). Mutations in vimentin can cause autosomal dominant cataracts, while those in BFSPs can be either dominant or recessive, with missense mutations tending to cause dominant cataracts, while nonsense mutations and frameshift causing deletions resulting in premature termination tend to cause recessive cataracts. Mutations in COL4A1 can cause dominant cataracts, and mutations in prolyl 3-hydroxylase 2 (P3H2, also known as LEPREL1), which is active in collagen chain crosslinking, can cause cataracts, sometimes accompanied by ectopia lentis and high myopia.

22.9 Chaperones and Protein Degradation

As lens fiber cells lack nuclei, they also lack protein synthesis and their proteins must last for the lifetime of the individual. In order to facilitate this, the lens contains high levels of chaperones such as the α-crystallins, although these also perform a more standard role as crystallin structural proteins in the lens. In this light, a mutation in UNC45B, a co-chaperone for HSP90 has been implicated in congenital cataract. Conversely, lens fiber cell differentiation also requires elimination of all organelles and their associated proteins, requiring highly active protein degradation systems. Mutations in *CHMP4B*, part of the endosomal sorting complex required for transport and autophagy, have been shown to cause autosomal dominant posterior polar or subcapsular cataract. Mutations in Ras-related GTP binding A (RRAGA), a component of the mTORC pathway, have been implicated in autosomal dominant cataracts. Mutations in the mitochondrial chaperone and protein degradation protease lon peptidase 1(LONP1) can also cause recessive cataracts, emphasizing the importance of mitochondrial function in the lens epithelia for lens transparency. *FYCO1 is* a scaffolding protein active in microtubule transport of lysosomes including autophagic vesicles. Mutations in *FYCO1* can cause autosomal recessive cataracts, consistent with an important role for autophagic vesicles in organelle degradation as equatorial epithelia differentiate into lens fiber cells. Interestingly,

all cataracts resulting from FYCO1 so far are nuclear. Finally, mutations in *EPG5*, a key regulator of autophagy that is active in autolysosome formation, while they have not been shown to cause isolated cataracts, do cause Vici syndrome, which includes cataracts [50].

22.10 Other Genes and Pathways

GCNT2 is the I-branching enzyme for poly-N-acetyllactosaminoglycans. In addition to determining the I (usually seen in children) and I (usually seen in adults) blood types it influences the epithelial-to-mesenchymal transition and cell migration, probably by influencing E-cadherin expression, and can cause autosomal recessive cataracts when mutated, about 50% of which are nuclear and 25% are lamellar and anterior polar, each. Mutations in *TAPT1*, which can disrupt Golgi structure and trafficking, can cause autosomal recessive cataracts, as can mutations in aldo-keto reductase family 1 member E2 (*AKR1E2*) and renalase (*RNLS*, FAD-dependent amine oxidase). Interestingly, mutations in the iron-responsive element of ferritin L (light chain, *FTL*) cause the hyperferritinemia-cataract syndrome in which loss of translational control results in massive overexpression of FTL that crystallizes in the lens and gives granular opacities in the nucleus and cortex. This example of an extraneous protein expressed at high levels in the lens emphasizes the requirement that crystallins or other proteins must be exceptionally soluble and stable to be expressed at crystallin-like levels without causing dysfunction. Finally, *TDRD7* is a widely expressed Tudor domain RNA binding and processing protein of RNA granules that also causes cataract when mutated, probably related to the high levels of mRNA synthesis required during lens differentiation.

22.11 Pathology

As mentioned above, cataracts have multiple causes, and thus present with different pathological findings. However, these can basically be grouped into two broad categories. Some congenital cataracts result from mutations with catastrophic effects on the protein, causing gross structural changes and precipitation or changes of similar impact in other lens components. The denatured proteins either escape or overwhelm binding by α-crystallin or other lens chaperones and are toxic to lens cells interfering with their proper differentiation and causing death and degeneration, often through the unfolded protein response (UPR) and apoptosis. These mutations are often associated with breakdown of the lens microarchitecture, including degeneration and perhaps calcification of lens fiber cells and eventually formation of large lacunae filled with proteinaceous debris with rupture of the lens capsule in the most severe cases. These cause large fluctuations in optical density with resultant light scattering. These are best studied in animal models of inherited congenital cataracts, with one example being a c.215+1G >A splice mutation in *CRYBA1* causing a p.Ile33_Ala119del mutant βA3/A1-crystallin protein [37], and many others also being well studied [36, 38, 51, 52].

In addition, light scattering and opacity will occur if there is a significant amount of high molecular weight (HMW) protein aggregates 1000 Å or more in size, even though the microarchitecture of the lens is well preserved [13, 14]. The short-range ordered packing of the crystallins, which must exist in a homogeneous phase for transparency, is important as is their stability over time. As increasing amounts of unstable mutant crystallins begin to denature and are bound by α-crystallins the size of the aggregates increases toward the 1000 Å limit. Eventually, the limit is passed, and light scattering begins, progressing to a clinically significant cataract when vision is impaired, although this mechanism appears to be more common in age-related cataracts. This can occur with normal lens histology, but eventually the α-crystallin is saturated, and HMW aggregates begin to come out of solution [34]. This can result in toxic effects on the lens fiber cells, with cellular degeneration and calcification, as seen in a rapamycin-induced model [53].

22.12 Clinical Features and Classification of Congenital Cataracts

Human cataracts can be classified using a variety of characteristics such as their age of onset, location in the lens, size, pattern or shape, density, and rate of progression. They can also be classified by their etiology, with about 30% of congenital cataracts in developed countries of genetic etiology, with most of the remainder idiopathic and a few percent due to intrauterine infection [9], although the fraction associated with infections and trauma can increase considerably in less developed nations [54]. Cataracts can also be classified by age at onset. Cataracts visible within the first year of life are generally considered congenital or infantile cataracts, the subject of this chapter. Juvenile cataracts are visible within the first decade of life, presenile cataracts are seen before the age of 45–55 years, and age-related cataracts with onset after 45–55 years.

Perhaps most usefully, cataracts can be classified by their appearance and anatomic location in the lens. The most commonly used system is that described by Merin, in which the cataract is classified as total (mature or complete), polar (including anterior or posterior), zonular (including nuclear, lamellar, and sutural), and capsular or membranous [55]. Since equatorial epithelia migrate laterally and then elongate and invert before moving into the nucleus in a concentrically ordered fashion during lens development, the location of a lens opacity can suggest the time at which the pathology initiated. When correlated the developmental expression of lens genes can suggest the genetic cause of the cataract. Nuclear opacities are likely to result from genes active during formation of the embryonic (months 1–3), fetal (months 3–9), or infantile (after birth), nucleus. Lens fiber cells continue to be laid down throughout life, so that lens opacities developing postnatally tend to present as cortical opacities or sometimes subcapsular opacities, which are also often associated with topical steroid drugs or radiation.

Polar opacities involve either the anterior (Fig. 22.2a) or the posterior (Fig. 22.2b) pole of the lens and may include the posterior subcapsular lens cortex (Fig. 22.2c) extending to the lens capsule. In addition to genetic causes, posterior subcapsular cataracts can occur secondarily to a variety of insults. Although posterior subcapsular cataracts have been associated with proliferation of Wedl cells (dysplastic bladder-like fiber cells) at least some posterior subcapsular cataracts are caused by abnormalities of the posterior fiber ends [46]. Polar opacities affecting both anterior and posterior poles are called bipolar. About 40% of Isolated anterior polar cataracts are caused by mutations in CRYAA and 30% of posterior polar cataracts are caused by mutations in *PITX3*, while 43% of posterior subcapsular cataracts are caused by mutations in *PITX3* and 29% by mutations in *GJA8*. (Table 22.3). Anterior polar cataracts are usually small, bilateral, and nonprogressive and do not impair vision. Anterior polar cataracts can be associated with microphthalmos, persistent pupillary membrane, or anterior lenticonus, while posterior polar cataracts can be associated with abnormalities of the posterior capsule including lentiglobus, lenticonus or with remnants of the tunica vasculosa. Although they are usually stable over time, they may progress, and can be associated with capsular fragility.

Nuclear cataracts show opacities in the fetal or fetal and embryonic lens nucleus (Fig. 22.2d, e). They can show a wide variation in severity, from dense opacities involving the entire nucleus to pulverulent (or dusty appearing) cataracts involving only the central nucleus or discrete layers (see below) and can be caused by mutations in a wide variety of genes.

Lamellar cataracts (Fig. 22.2f, g) affect lens fibers, which are formed at the same time, resulting in a shell-like opacity at the level at which the fibers were laid down at the time of the presumed insult. They are the most common type of congenital cataract and can be caused by a wide variety of genes (Table 22.3). Some cataracts have associated arcuate opacities within the cortex called cortical riders (Fig. 22.2g).

Sutural or stellate cataracts (Fig. 22.2h, i) affect the regions of the fetal nucleus on which the ends (or feet) of the lens fibers converge, called the Y sutures. Even in normal lenses, the sutures are visible by slit lamp biomicroscopy

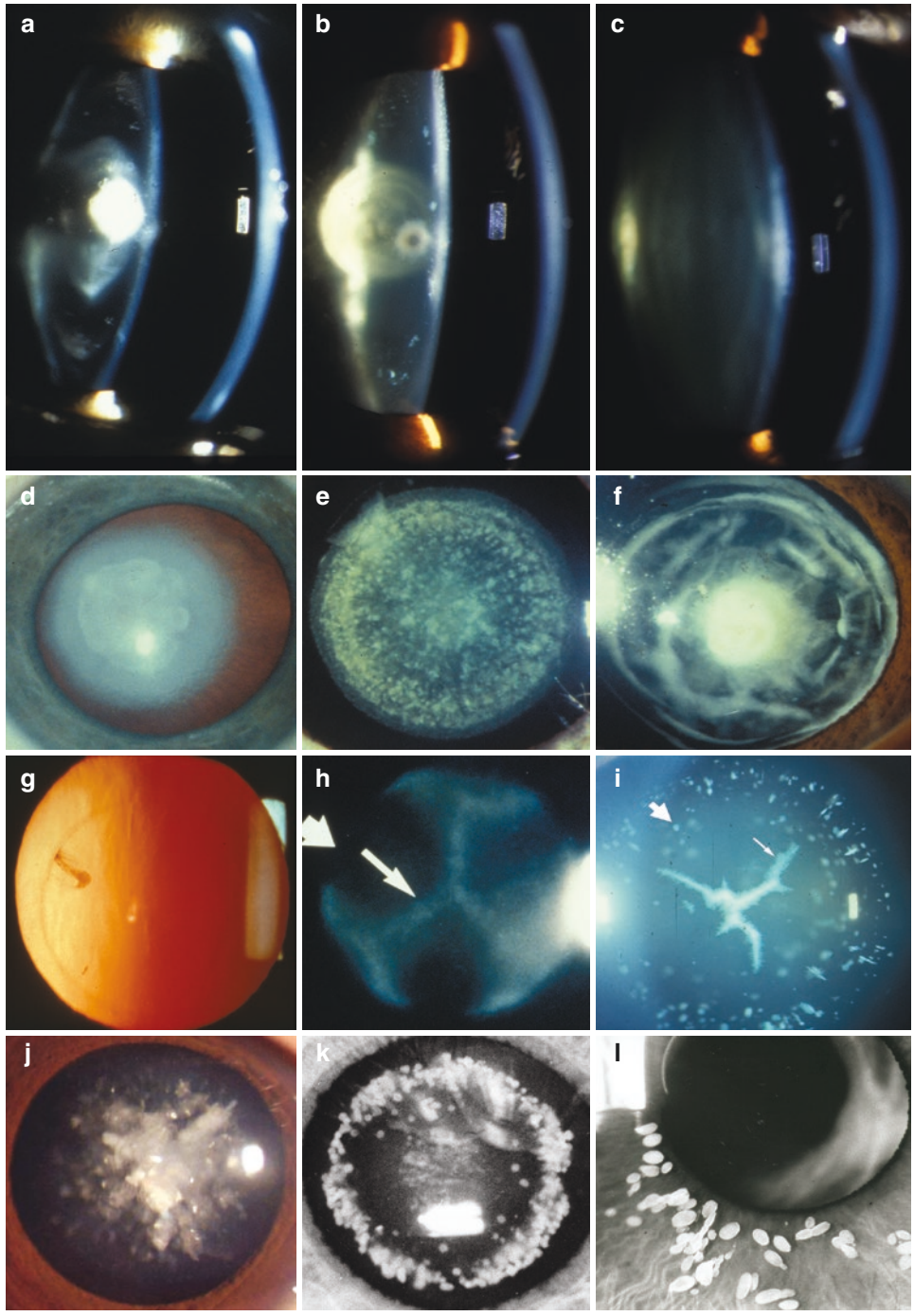

Fig. 22.2 Examples of cataract morphologies. (**a**) Dense anterior polar cataract visible on slit lamp examination. Some opacification of the lens nucleus is also visible. (**b**) Dense posterior polar cataract is visible on slit lamp examination. A smaller anterior polar cataract is also visible so that this would be termed a bipolar cataract. (**c**) Posterior subcapsular cataract. (**d**) Dense nuclear cataract. The macula and optic nerves are obscured by this cataract. (**e**) Punctate nuclear cataract. (**f**) Multi-lamellar cataract with an anterior polar component. (**g**) Very fine nuclear lamellar pulverulent cataract viewed by retroillumination with a cortical rider at 10 o'clock. (**h**) Sutural cataract with a nuclear lamellar component. (**i**) Sutural cataract with a cortical cerulean or blue dot component. (**j**) Corraliform cataract (courtesy of Li et al. [67]). (**k**, **l**) Ant's egg cataract (courtesy of Hansen et al. [56])

Table 22.3 Fractions of cataract types caused by specific genes

	nuclear	lamellar[a]	sutural	cortical	PP[b]	AP[c]	corralliform	cerulean	PSC
GJA8	0.09	0.12	0.05	0.00	0.06	0.00	0.00	0.00	0.29
GJA3	0.10	0.16	0.05	0.02	0.06	0.00	0.16	0.00	0.00
CRYAA	0.12	0.12	0.03	0.02	0.06	0.40	0.00	0.00	0.00
CRYAB	0.02	0.03	0.00	0.00	0.13	0.00	0.00	0.00	0.00
CRYBB1	0.06	0.00	0.03	0.05	0.03	0.00	0.00	0.00	0.00
CRYBB2	0.04	0.05	0.03	0.07	0.00	0.00	0.05	0.43	0.14
CRYBB3	0.01	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00
CRYBA3	0.08	0.10	0.19	0.07	0.03	0.00	0.00	0.00	0.00
CRYBA4	0.01	0.03	0.00	0.00	0.00	0.10	0.00	0.00	0.00
CRYGC	0.08	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
CRYGD	0.09	0.04	0.03	0.02	0.06	0.10	0.74	0.21	0.00
CRYGS	0.00	0.04	0.05	0.07	0.00	0.00	0.00	0.00	0.00
NHS	0.05	0.00	0.30	0.12	0.00	0.00	0.05	0.00	0.00
HSF4	0.02	0.12	0.03	0.10	0.00	0.10	0.00	0.00	0.00
EPHA2	0.05	0.01	0.00	0.14	0.06	0.10	0.00	0.00	0.14
FOXE3	0.01	0.00	0.00	0.07	0.00	0.00	0.00	0.21	0.00
MAF	0.02	0.04	0.00	0.00	0.09	0.10	0.00	0.07	0.00
PITX3	0.00	0.00	0.00	0.02	0.38	0.00	0.00	0.00	0.43
EYA1	0.01	0.01	0.00	0.02	0.00	0.00	0.00	0.00	0.00
BFSP2	0.01	0.03	0.14	0.07	0.00	0.00	0.00	0.00	0.00
AQP0	0.04	0.04	0.08	0.07	0.03	0.00	0.00	0.07	0.00
CHMP4B	0.01	0.01	0.00	0.00	0.00	0.10	0.00	0.00	0.00
FYCO1	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Frequencies are calculated from CAT-MAP
[a]Lamellar or zonular
[b]Posterior polar
[c]Anterior polar

as an upright Y anteriorly and an inverted Y posteriorly. About 30% of sutural cataracts result from mutations in NHS, while the remainder are caused by multiple additional genes, with 19% associated with mutations in CRYBA3 and 4% with mutations in BFSP2 (Table 22.3). Cerulean, or blue dot cataracts are characterized by numerous small bluish opacities in the cortical and nuclear areas of the lens (Fig. 22.2i). About 43% of cerulean cataracts are caused by mutations in CRYBB2 while another 21% are caused by mutations in CRYGD and FOXE3 each (Table 22.3). Coralliform cataracts are dispersed popcorn or coral-like cataracts primarily in the nuclear area (Fig. 22.2j). About 74% of coralliform cataracts are caused by mutations in CRYGD, with about 16% caused by mutations in GJA3 (Table 22.3). Other varieties of cataract can usually be described through a combination of the above terms, although there are some specialized cataracts that have unique characteristics, such as the ant's egg cataract (Fig. 22.2k, l), in which a mutation in connexin 46 causes beaded structures like ants eggs to form from the lens [56, 57].

Mature or total cataracts may represent a late stage of any of the above types of cataracts, in which the entire lens is opacified. Membranous cataracts result from resorption of lens proteins, often from a traumatized lens, with resulting fusion of the anterior and posterior lens capsules to form a dense white membrane. They usually cause severe loss of vision.

22.13 Genetic Aspects of Congenital Cataracts

As has been mentioned above, about 85% of inherited congenital cataracts show an autosomal dominant inheritance pattern, although this varies significantly depending on the population and study (Table 22.2b). In addition, there

is a significant variation in inheritance patterns among the various genes. All cataracts caused by CRYBB2, CRYBA3, CRYGC, CRYGD, CRYGS, and MAF are dominant, which suggests that there might be redundant biological systems for these proteins in the lens so that their absence by itself would not disrupt lens biology and transparency. In contrast, the presence of autosomal recessive inheritance patterns of cataracts caused by CRYBB3 and CRYBA4 suggests that they might have an irreplaceable role in lens biology in addition to that of structural lens crystallins. In contrast, the absence of autosomal dominantly inherited cataracts resulting from GCNT2 and FYCO1 suggests that these cataracts all result from the absence of the functional protein, implying a unique and necessary role for these genes in the lens.

22.14 Clinical Aspects of Congenital Cataracts

Cataracts that interfere with vision significantly, require early diagnosis and prompt evaluation to determine their etiology if possible. As one example, treatment of galactosemia in early life will permit recovery of the lens to normal clarity. Conversely, if lens clarity is significantly compromised, surgical treatment might be required. However, in general, severe congenital cataracts require surgical treatment to allow the functional retinal–cortical connections required for vision to form successfully.

Because unequal ocular input into cortical neurons due to unilateral form deprivation results in more severe visual deficits than does bilateral deprivation [58–60] a unilateral dense congenital cataract is generally considered to be a surgical emergency while bilateral dense cataracts allow more routine scheduling. Thus, unilateral dense cataracts can be operated successfully in the first weeks of life, while bilateral cataracts can be operated successfully until 3 months of age. With prompt surgery, the visual prognosis is better for bilateral as compared with unilateral cases and in less dense cataracts as compared with total opacities. Chronic dilation of the pupil in small centrally located congenital cataracts, allowing the infant to see around the cataract, may be useful in some cases when cataract surgery may not be immediately feasible. When congenital cataracts are associated with other ocular abnormalities and/or systemic disease, a poorer visual outcome often results [60–62]. Finally, it should be emphasized that communication between clinicians, therapists, and teachers combined with counseling of patients is very important in the treatment of young cataract patients and their families [63]. More recently, there has been much interest in small molecule chaperones that might stabilize or even renature damaged crystallins [49, 64], although these would probably be more relevant for treatment of age-related cataracts. Finally, promising results have been obtained by using lens regeneration rather than inserting an intraocular lens, although this approach is still highly experimental [65].

22.15 Molecular Biology of Congenital Cataracts

As described briefly above, congenital cataracts tend to result from mutations with severe functional consequences for the mutant protein structure and function and are often accompanied by significant disarray of the lens microarchitecture, as shown in a number of model systems [36–38, 51, 52]. This breakdown in lens microarchitecture is usually accompanied by induction of the unfolded protein response with subsequent activation of apoptotic processes. This pathological process contrasts with that seen in most age-related cataracts, which are characterized by increased sensitivity of mutant or variant proteins being acted on by environmental factors to give a gradual decrease in stability followed by denaturation and binding by α-crystallin [66]. These two mechanisms are not exclusive, as potentially toxic high molecular weight protein aggregates can form when the lens cell α-crystallin becomes saturated with denatured crystallins, resulting in damage to lens cells.

22.16 Laboratory and Clinical Evaluation of Congenital Cataracts

Cataracts may be examined clinically in a variety of ways. Looking at the pupil with a handlight, will show a white opacity (termed leukocoria). Direct ophthalmoscopy can suggest the effect of the cataract on visual function since sharp visualization of retinal components such as the optic nerve and macula suggest that the patient can see out as well. In addition, a lens opacity can be silhouetted in the red reflex using either direct or retroillumination. However, a more definitive description of the lens opacity requires slit lamp biomicroscopy pupillary dilation, allowing both direct and retroillumination with magnification sufficient to visualize the lens opacity and define its morphological features.

After establishing the significance and classification of the cataract by type, the evaluation of a cataract consists of a careful assessment of its effect on the visual acuity and function. In very young children from 0 to 3 years old observation-fixing, following, covering alternative eyes, and observing the response are useful. If more accurate evaluation is required, visually evoked cortical responses, preferential looking, or the forced choice method may be used. As children grow older, the illiterate E or Allen cards using picture differentiation can be used, and once the child has mastered the alphabet a logEDTRS or Snellen chart may be used.

Not all congenital cataracts are genetic in origin, with perhaps the most common differential diagnosis being prenatal infections by viruses or other infectious diseases. Of these, rubella directly involves the lens while other infectious diseases such as toxoplasmosis, mumps, measles, chickenpox, herpes simplex, herpes zoster, influenza, echovirus type 3, and cytomegalovirus, cause uveitis (ocular inflammation). A good screen for these diseases is TORCH titers. Developmental disorders due to prematurity, with birth anoxia, low birth weight, central nervous system involvement characterized by seizures, cerebral palsy or hemiplegia, and retinopathy of prematurity. Multisystem syndromes including chromosomal abnormalities can be suggested by the clinical examination and tested by chromosome analysis or blood and urine chemistries specific for the disorder suspected. Some perinatal–postnatal problems such as hyperglycemia (associated with signs of diabetes) and hypocalcemia (usually characterized by tetany), can cause cataracts and can be detected using serum chemistries. Finally, cataracts may be associated with other ocular abnormalities including anterior chamber abnormalities such as Reiger syndrome, primary hyperplastic vitreous, and aniridia, or with retinopathies such as retinal dysplasia, Norrie disease, and microphthalmia.

22.17 Summary

Inherited congenital cataracts affect all populations throughout the world and are a significant cause of blindness in infants that require early diagnosis and prompt treatment. While clinically identical cataracts can be caused by mutations in different genes and identical mutations in the same gene can cause clinically different cataracts, it is possible to identify general correlations between some of the causative genes and specific cataract morphologies, which might be useful in guiding genetic diagnosis. Genes associated with congenital cataracts tend to belong to molecular or biochemical pathways important for lens development and homeostasis. While we have identified many genes, there remains much work to be done both in identifying the remaining causative genes and in understanding the molecular pathologies that lead to the common endpoint of lens opacity or cataract.

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23 Higher Order Aberrations: Differences Among Populations from Various Demographics

Gaurav Prakash

Abstract

The understanding of the role of higher order aberrations of the visual system has explained multiple optical phenomena to the researchers and clinicians alike. Many disorders are better understood now due to application of wavefront optics in ophthalmology. In this chapter, we first summarize the current basic understanding of higher order aberrations and then review the literature on the differences in the normative data from various demographic populations in the higher order aberrations noted. A pooled analysis of the data suggested that in most of the demographic databases, at a 6-mm wavefront diameter, the adult human eye tends to have anywhere between 0.3 and 0.4 μm of higher order aberrations root mean square (HOARMS). However, there were variations noted in sub-analysis of the Zernike modes between different populations. The role of normative data for a given population is that of a guideline. It gives base information on which individual wavefront profile of an eye can be evaluated for the differences between normal and abnormal.

Keywords

Higher order aberrations · Normative data Wavefront optics · Demographic · Population variation · Coma · Spherical aberrations Aberrometry · Asian · Caucasian Middle-Eastern

23.1 Introduction

The measurement of refractive error beyond the conventional spherocylinder is called ocular aberrometry [1–3]. It has applications in multiple conditions such as refractive surgery, corneal pathology including scarring and ectasia, effects of crystalline and artificial lenses on vision, lesions causing corneal traction or distortion, amblyopia, and even adnexal disorders such as ptosis [1–8]. As wavefront optics is a highly complex and mathematical topic, sometimes clinicians avoid the details. Therefore, we will first cover some basics of the subject and then discuss the demographic variations.

G. Prakash (✉)
University of Pittsburgh,
Pittsburgh, United States

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23.2 Basics of Ocular Higher Order Aberrations

Ocular aberrations are measured with devices called aberrometers [1–3]. Most commonly, aberrometers are based on Hartmann shack principle; the essential premise being the measurement of difference between a distorted wavefront and a normal one (Figs. 23.1 and 23.2) [1–3]. Once these differences are measured, they are broken down mathematically into smaller units (decomposed) from a larger wavefront of light using polynomials (Zernike polynomials) or analyzed as interfering wavelets (Fourier analysis) [1–3, 9]. The total distortion is conventionally regrouped as lower and higher order

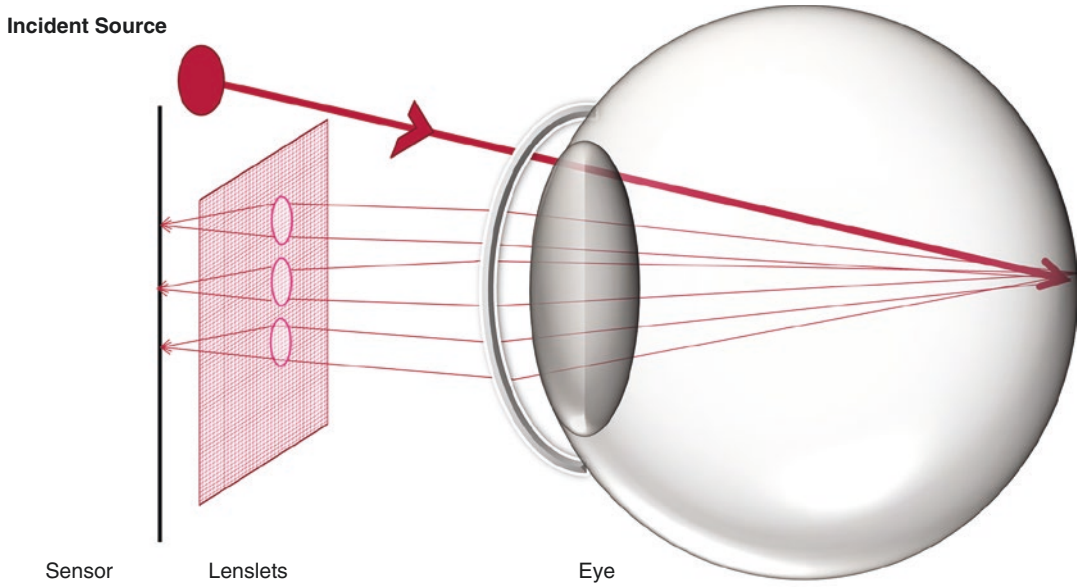

Fig. 23.1 Principle of Hartmann Shack Aberrometry

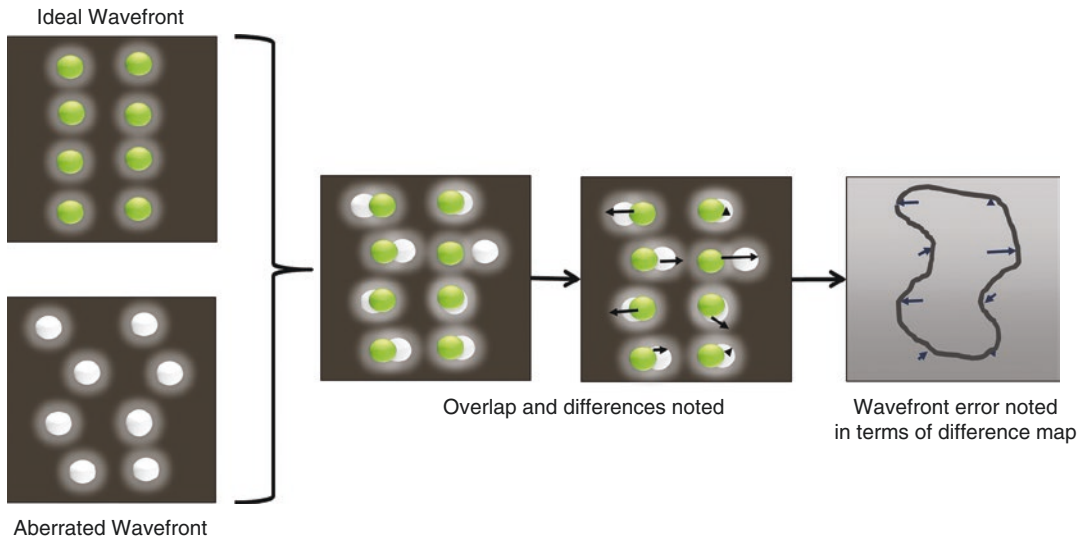

Fig. 23.2 Wavefront error estimation from the data derived from aberrometer

aberrations. The lower order aberrations approximate the sphero-cylinder refractive error and are mathematically represented till the second order. Beyond the second order, aberrations are conventionally called as higher order aberrations. Common names for some of the higher order aberrations include coma, spherical aberrations, and trefoil. Coma and spherical aberrations are particularly more symptomatic and are often seen in clinical settings (Fig. 23.3). As compared to sphere and cylinder which has a single plane of focus or a maximum of two meridians of primary power, higher order aberrations have a higher number of planes of focus and more meridians of power. This makes correcting wavefront error with uniplanar glasses impractical. As long as the component of higher order aberrations is smaller in the overall refractive error, patients are not symptomatic. The possible explanation for this phenomenon is the adaption of the visual system to existing aberrations. Sometimes the aberration profile of the eye is altered, due to surgical, traumatic, or progressive change. This decompensated profile of aberrations becomes intolerant to the visual system. These cases typically do not improve fully with refraction and even if they do, the visual quality does not match the quantity of vision. A more detailed, nonmathematical discussion on the application of wavefront optics can be found elsewhere [3].

23.3 Factors Affecting Wavefront Capture

The technician's learning curve and the ability of the patient to relax and focus on the fixation target are essential. It should be noted that wavefront diameter at reporting or capture is the most important factor governing the amount of aberrations noted. For this precise reason, it is a universal practice nowadays to mention the pupillary

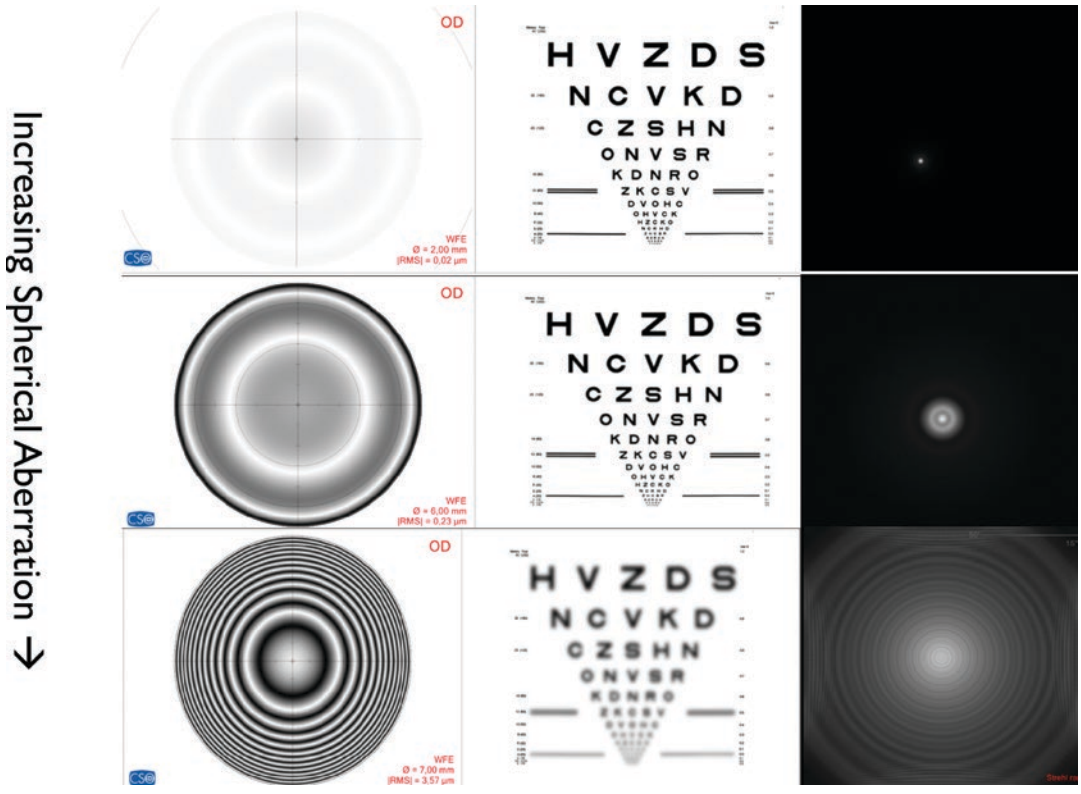

Fig. 23.3 Optical effect of increasing spherical aberration

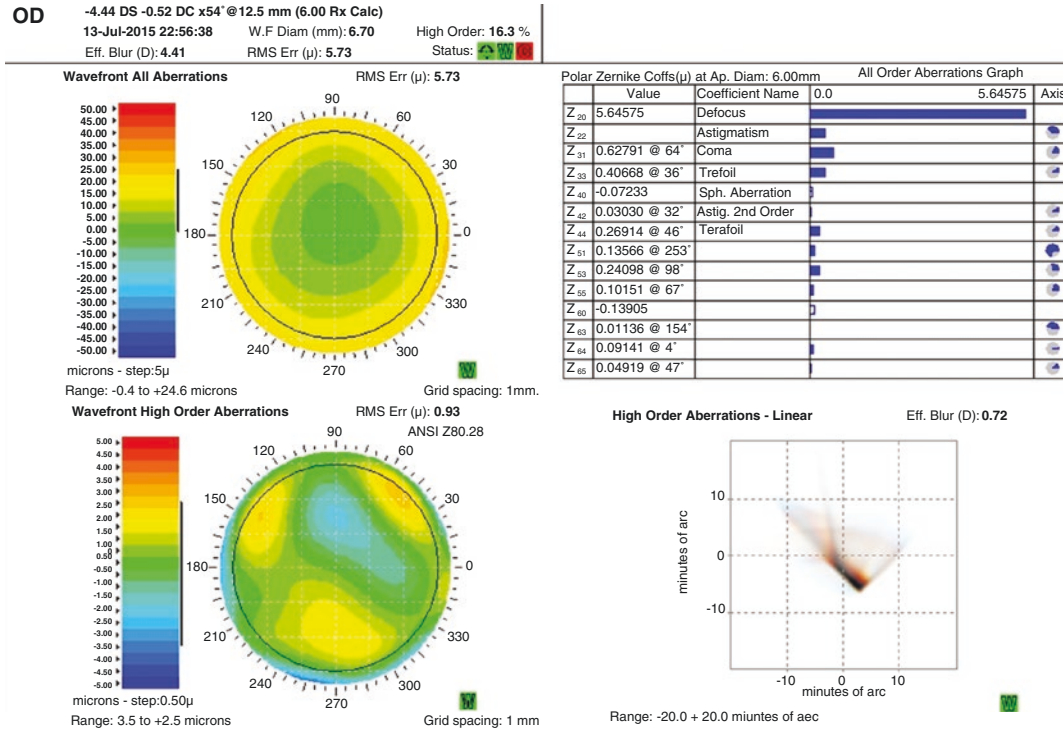

Fig. 23.4 Representative Aberrometer printout with the wavefront diameter, higher order and total aberrations, individual Zernike modes and other information

diameter when reporting or comparing wavefront data. A good quality aberrometric reading and the interpreter's familiarity with the specific data given by the aberrometer are also very important (Fig. 23.4). Researchers often report wavefront data for the whole eye at 6 or 4 mm and for the cornea at 8 or 6 mm. Roles of tear film stability, dilatation drops, and other medications are also important. Therefore, a good quality wavefront data from a normative population should be non-diseased candidates, and the best set of data is often from refractive surgery candidates who have already been screened and noted as good candidates for refractive surgery.

Age of the person is also one of the governing factors for the amount and the type of higher order aberrations found. Many studies have noted a positive correlation between the age and the amount of higher order aberration [10–15]. When a person ages, a change in the spherical aberration profile is seen. A possible logical explanation is the lenticular change with age. However, there seem to be additional factors such as corneal spherical aberrations because the trend for increase in spherical aberration with age has also been seen in children [11–14]. It is important to evaluate the data from ocular wavefront between two normal cases with the added perspective of the age difference between them.

23.4 Need for Normative Databases of Higher Order Aberrations

With the vast experience of ophthalmic practitioners with conventional spherocylinder, there is a strong intuitive idea about normal refractive error ranges. For example, many practitioners consider a refractive error of –5 or more diopters "high" [16]. Some populations have higher incidence of refractive errors compared to others [17]. Ophthalmologists, optometrists, and ophthalmic technicians based out of the populations

with higher refractive errors are aware of these variations and treat their patients accordingly. However, a lack of similar data for higher order aberrations was a major factor hampering comparative work in ophthalmic wavefront optics and evaluating the normative ranges. Therefore, many researchers and ocular scientists studied the normative data for different populations for higher order aberrations in order to create awareness on demographic variations and also to compare pathology with normal. In this chapter, we will summarize the major studies done to evaluate normative wavefront population and summarize the currently existing knowledge.

23.5 Population-Based Variations in Higher Order Aberrations

23.5.1 Caucasian/American Demographics

The earlier studies on the normative data of ocular wavefront were from the United States. Two such studies were by Wang and Koch [18] and by Netto, et al. [19]. Wang and Koch evaluated 532 eyes of 306 subjects and found that for a 6.0 mm pupil mean higher order aberration root mean square (HOARMS) was 0.305 ± 0.095 μ for a mean refractive error of –3.34 ± 2.8 diopters with the WaveScan system (Visx, Inc) [18]. Netto et al., evaluated 226 consecutive refractive surgery candidates (418 eyes) with the WaveScan (VISX, Santa Clara, Calif). At a 6-mm pupil size, they noted that the HOARMS were 0.23 ± 0.11 μ for a mean refractive error of –3.40 ± 3.14 diopters [19]. Both these studies found similar results for normal population in a US-based setting at similar refractive errors and same pupil size (As aberration magnitude is pupil dependent, it is important to use the similar pupil size when comparing two wavefront data). By the next year (2006), a large database of meta-analyzed Hartmann-Shack data for 2560 eyes pooled from 10 centers (8 in the United States, 1 each in Japan and Spain) was published [20]. This data provided comprehensive statistical limits for normative population data. At a 6-mm pupil, the mean HOARMS value was 0.33 ± 0.13 μ in this study. The authors noted that there was a general consensus for the magnitude of HOAs expected in normal adult human eyes. At least 90% of the samples had aberrations less than double the mean values, suggesting the authors' conclusion that these values can serve as a set of reference norms [20].

So far, it was established that higher order aberrations fall within a narrow mean range. However, as noted, this data was predominantly from the United States and was largely for the Caucasian population. However, later studies addressed other demographics and also compared the data with existing literature.

23.5.2 Asian Population (Chinese and Indian Subcontinental)

In the year 2006, a study from Brazil noted that even though Asian-origin patients have a higher prevalence of myopia than non-Asian origin patients, there were no differences in the higher order aberrations. Both the data sets had an aberration profile of HOARMS mean ~0.5 μ [21]. Another set of data on Chinese eyes by Wie et al. [22] had mean HOARMS at 6.0 mm pupil of 0.49 ± 0.16 μ for a mean refractive error of sphere –5.23 ± 1.79 D and cylinder –1.29 ± 0.98 D. Prakash et al. did the first study of normative data from India. Two hundred six candidates (412 eyes) of patients with North Indian origin were evaluated in this study for multiple wavefront parameters including 6-mm HOARMS. The data was then compared to previously published Caucasian and Chinese data. For a mean refractive error of –2.97 ± 4.0 D sphere and 1.73 ± 3.6 D cylinder the mean 6.0 mm HOARMS for the Indian population was 0.36 μ ± 0.26 μ. Third- and fourth-order aberrations were between 60 and 70% higher in Asian (Chinese) eyes (from already published data). The authors concluded that the normative data for HOARMS in Indian eyes closely matched that in white populations but was different from that in Asian (Chinese) populations [23]. In another comparative study, Cervino et al. found that the HOARMS values for

Caucasian and British Asians were similar. With the exception of Z33 and Z40, all other Zernike modes in their study were comparable [24].

23.5.3 Middle Eastern Population

In a study from Iran, 904 eyes of 577 people were analyzed. The mean HOARMS in this study was 0.306 μ [15]. In another study, Prakash, et al. compared the higher order aberrations in Arab eyes and South Asian eyes from a single setting in Abu Dhabi, United Arab Emirates [25]. Two hundred Arab participants (group 1) and 200 participants of South Asian origin (group 2) comprised the study sample. The mean of the HOARMS was 0.36 ± 17 μ in the Arab group, which was similar to the South Asian group.

A unique advantage of comparative studies using the same setting for two different ethnic populations is that it rules out machine and operator variations. However, compared to larger pooled data, these studies tend to have a smaller sample size.

23.6 Conclusion

As we noted, most studies have found that the population means of HOARMS at 6 mm for refractive error candidates is the range of 0.03–0.4 μ. This trend is seen across Caucasian, Indian (Asian), Chinese (Asian), and Middle Eastern populations. Sub-analysis sometimes reveals subtle differences between these groups; however, as the overall normative range is the same, a fair idea can be made on the values expected. It should be noted that higher order aberrations are fairly unique method of assessment of refractive error. Two patients with similar sphero-cylindrical refractive error may have different wavefront profile. Therefore, it is important to compare the patient's wavefront data with his previous records on postoperative follow-up and with the fellow eye in unilateral pathology. The role of individual variations is surely greater in terms of direct clinical care. However, an understanding of the population normally gives the clinician and the researcher a perspective into the narrow band of data beyond which a variation should not be expected, and should be looked for causes.

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Genetics of Microphthalmia: Global and Indian Perspectives

24

Sankaranarayanan Rajkumar,
Vidya Gopinathan Nair,
and Abhay Raghukant Vasavada

Abstract

Globally, visual impairment and blindness in children are important problems as they affect one's quality of life. Clinical anophthalmia and/or microphthalmia, often used interchangeably, are congenital eye anomalies that constitute a major proportion of childhood blindness. These rare disorders are often associated with other birth defects that could bring negative impact and eventually lead to human morbidity and social burden by means of loss of workforce and economy. Therefore, in order to bolster some preventive strategies and to reduce the burden of visual impairment, understanding the genetic and pathological mechanisms of congenital ocular anomalies holds priority. In this chapter, we attempted to describe the epidemiology, etiology, and the molecular genetic mechanisms of normal eye development and microphthalmia. Further, we discussed genetic variations involved in disease phenotype with respect to Indian and global perspective.

Keywords

Congenital ocular anomalies
Microphthalmia · Epidemiology · Etiology
Genetics · Molecular mechanisms

24.1 Introduction

> Of all the senses, sight must be the most delightful—Helen Keller

A group of eye diseases and conditions that occur in childhood or early adolescence often results in blindness or severe visual impairment if left untreated [1]. Although the prevalence varies (0.3–1.5 per 1000 children) in different regions of the world according to socioeconomic condition, an estimate revealed that approximately 1.4 million of children are blind globally and three-quarters of them live in the poorest regions of Africa and Asia [2–4]. Childhood blindness has far-reaching implications for the affected child and family, and throughout life profoundly influences educational, employment, personal, and social prospects [5]. The global financial cost of childhood blindness in terms of loss of earning capacity (per capita gross national product (GNP)) is greater than the cost of adult blindness and has recently been estimated to be between US$6000 million and $27000 million [6].

S. Rajkumar (✉)
Department of Ophthalmic Genetics, Aditya Jyot Foundation for Twinkling Little Eyes, Mumbai, Maharashtra, India

V. G. Nair · A. R. Vasavada
Department of Pediatric Ophthalmology, Iladevi Cataract and IOL Research Centre, Ahmedabad, Gujarat, India
e-mail: icirc@abhayvasavada.com

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These financial costs alone, however, provide only one perspective of the public health burden of blindness. Thus, the control of childhood blindness has been identified as a priority of the World Health Organization's (WHO) global initiative for the elimination of avoidable blindness by the year 2020 [7].

Microphthalmia alone constitutes about 3.5% of childhood blindness (Fig. 24.1). Clinical anophthalmia and microphthalmia contribute 1 per 30,000 and 1 per 7000 live births, respectively [18]. However, different studies have reported different prevalence and incidence among different countries (Table 24.1). Microphthalmia occurs either in isolation or as a part of another syndrome [19]. Microphthalmia is highly heterogeneous and includes a variety of developmental eye deformities such as aniridia, iris hypoplasia, microcornea, coloboma, aphakia, sclerocornea, glaucoma, optic nerve atrophies, corneal and retinal dystrophies, and others [20–24]. They are also associated with extraocular features such as brain, skeletal, craniofacial, facial, cardiac, genital, skin, renal, and other abnormalities [23, 25–29].

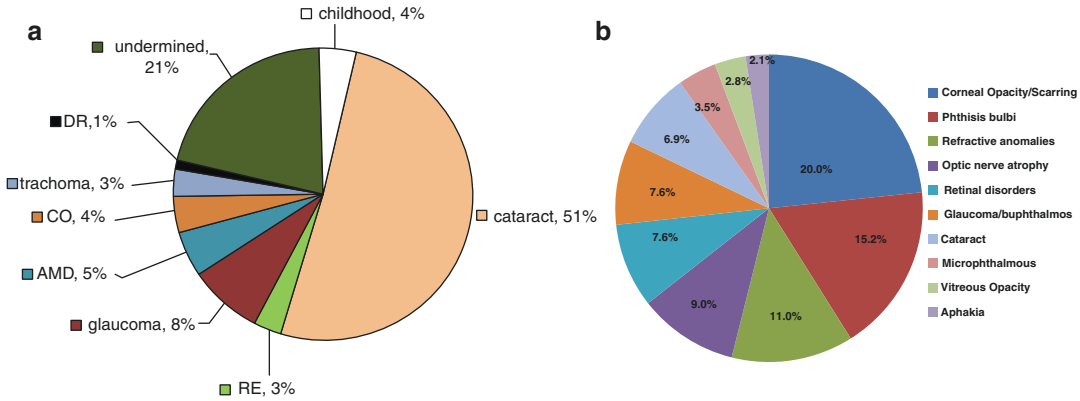

Fig. 24.1 Global causes of blindness (**a**) and childhood blindness (**b**). Figure 24.1a is reproduced with permission from the World Health Organization [*ID No. 326793*]. Mariot, SP Global Data on Visual Impairments 2010, published by the World Health Organization (WHO/NMH/PBD/12.01) (https://www.who.int/blindness/GLOBALDATAFINALforweb.pdf)

Table 24.1 Prevalence estimates of A/M from various population-based studies

Country/Location	Duration	Ethnicity	Per 10,000 births	References
USA, Atlanta	NA	American-African-Caucasians	2.56	[8]
USA, California	1989–1995	American-African-Caucasians	0.40	[9]
USA, Hawaii	1986–2001	American-African-Caucasians	3.21	[10]
USA, Texas	1999–2009	American-African-Caucasians	3.00	[11]
Sweden	1965–2001	Caucasians	1.36	[12]
France	1979–2004	Caucasians	2.50	[13]
Spain	1980–1995	Caucasians	2.13	[14]
Italy	1981–1989	Caucasians	0.60	[15]
Denmark	1995–2012	Caucasians	1.51	[16]
United Kingdom	2006–2008	Caucasians	0.63	[17]
England-Wales	NA	Caucasians	0.22	[8]

NA Data on duration of survey is not available

24.2 Clinical Diagnosis and Classification of Microphthalmia

24.2.1 Diagnosis of Microphthalmia

It involves a gross clinical examination and imaging techniques.

24.2.1.1 Gross Clinical Examination

(a) Looking for evidence of globe, palpation of the orbit to obtain an estimate of globe size.
(b) Measurement of corneal diameter.

24.2.1.2 Imaging Techniques

(c) A scan ultrasonography to measure total axial length and length of anterior and posterior segments.
(d) B scan ultrasonography to evaluate the internal structures of the globe.
(e) Computerized tomography scans (CT scans) and magnetic resonance imaging (MRI) of the brain and orbits (orbits to evaluate the size and internal structures of the globe, presence of optic nerve and extraocular muscles, and brain anatomy). Although the globe is inconspicuous on clinical examination, CT or MRI reveals remnants of ocular tissue, an optic nerve, and extraocular muscles. Without orbital imaging studies, severe microphthalmia can be mistaken for anophthalmia.

24.2.2 Classification of Microphthalmia

Based on the anatomic appearance of the globe and severity of axial length reduction, microphthalmia is classified as.

2.2.1. Total microphthalmia	• Microphthalmia refers to the presence of a hypoplastic or rudimentary eye inside the orbit. • Globe having total axial length at least two standard deviation below the mean for age. • Characterized by foreshortening of both anterior and posterior segments.
2.2.1.1. Congenital cystic eye	• Characterized by the presence of cyst and no trace of the globe in the orbit owing to the failure of invagination of the optic vesicle.
2.2.1.2. Anophthalmia	• True anophthalmia is rarely compatible with life. Therefore, anophthalmia is described as clinical anophthalmia or severe microphthalmia. • According to medical literature, anophthalmia refers to the complete absence of the eye (or globe) but with the presence of ocular adnexa (conjunctiva, eyelids, and lacrimal apparatus). • The globe is severely reduced in size, with a corneal diameter <4 mm and a total axial length <10 mm at birth or <12 mm after 1 year of age.
2.2.1.3. Simple microphthalmia	• Described as nanophthalmia, the eyes are usually deeply set in the orbit, refraction is high hypermetropic, and the cornea is small. • Both anterior and posterior segments are foreshortened, the relative lens volume is increased, and there is sclerochoroidal thickening.
2.2.1.4. Microphthalmia with intraocular malformations	• Microphthalmia with anterior chamber malformations includes dysgenesis of the cornea, iris, iridocorneal angle, ciliary body, and congenital cataract (as in sporadic Hallermann–Streiff syndrome and X-linked Nance–Horan syndrome). • Microphthalmia with posterior chamber malformations includes persistent hyperplastic primary vitreous, coloboma of the uvea, optic nerve, chorio-retina, cystic coloboma, and retinal dysplasia).
2.2.1.5. Microphthalmia with multiple ocular malformations	• Characterized by malformations derived from different embryological germ layers as in the oculocerebrocutaneous syndrome (Delleman syndrome) and osteoporosis-pseudoglioma syndrome.
2.2.2. Partial microphthalmia	• Either the anterior or posterior chamber will be in normal size when the opposite chamber is small.

24.3 Etiology of Microphthalmia

Microphthalmia is extremely heterogeneous phenotype and displays both unilateral and bilateral symptoms with uneven penetrance [19]. Although the pathology and disease mechanism of microphthalmia remains unknown, the role of environmental and infectious agents and genetic factors is suggested.

In the early 1990s, the UK reported a possible association of microphthalmia conditions with the use of pesticide Benomyl, but later it was refuted by other studies [30–33]. Gestational-acquired infections such as rubella, toxoplasmosis, cytomegalovirus, and varicella [33, 34] and other viruses of the herpes-zoster family, parvovirus B19, influenza virus, and coxsackie A9 [35, 36] and gestational diabetes are also considered to be associated with microphthalmia condition. Maternal vitamin A deficiency [37], fever, and exposure to X-rays, hyperthermia, solvent misuse, and exposure to drugs like warfarin, thalidomide, and alcohol [33] are also linked to microphthalmia.

The genetic factors accountable for microphthalmia comprise chromosomal defects, copy number variations, and genetic mutations. Among the chromosomal defects, trisomy 9, trisomy 13, trisomy 18 [38], chromosomal deletions such as 4p-, del7p15.1-p21.1, 13q-, ring13, delXp22.3, del 14q22.1q23, 18q-, del3q26 [39–41], and chromosomal duplications such as dup3q26, dup3q21-ter dup, dup4p, and dup10q have been reported. Several genetic loci and mutation in candidate genes have also been reported. Until now about 82 genes are reported to be involved in the pathogenesis of microphthalmia [18], and transcription factors represent the largest group, followed by TGF-β/BMP signaling molecules, retinoic acid pathway genes, and other genes with known or unknown functions [42, 43]. Genetic mutations giving rise to microphthalmia could be inherited as autosomal dominant, autosomal recessive, and/or X-linked [38].

24.4 Molecular Genetic Mechanism of Normal Eye Development

24.4.1 Formation of Eye Field

The eye formation starts with the specification of the eye field (Fig. 24.2a–g). During gastrulation, the formation of the neural plate (Fig. 24.2a) from dorsal ectoderm (neural epithelium) is the initial stage of eye field programming (Fig. 24.2g). It is regulated by secreted signaling molecules from neural epithelium such as fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), Wnts, transforming growth factor β (TGF-β) and bone morphogenetic protein (BMP), and transcription factors (Rx, Pax6, Otx2). Although diverse molecular signaling factors and their antagonists are implicated in the specification of the eye field, inhibition of BMP signaling by ventroptin (a BMP antagonist secreted by Spemann Organizer) is one of the crucial steps in this process [44–49]. In addition, the coordinated expression of Rx, Pax6, Six3, Lhx2, Six6/Optx2, ET, and *tII* appears to be essential for the specification of the eye field [50–54].

24.4.2 Patterning of Neural Plate

Patterning of neural plate into distinct subdomains, antero-posteriorly, namely the forebrain (or prosencephalon), midbrain (or mesencephalon), hindbrain (or rhombencephalon), and spinal cord, is the second step toward eye field separation and is regulated by FGFs, retinoic acid, and Wnts [55–58]. The forebrain patterning is the preceding step in the event of eye field separation, which is precisely regulated by Wnt/β-catenin signaling, HesI and Otx2, promoting diencephalic at the expense of telencephalic/eye field fates [53, 59, 60]. Further, the interplay between the non-canonical Wnt pathway and BMP influences the establishment of a boundary between telencephalon and eye field [reviewed by 61].

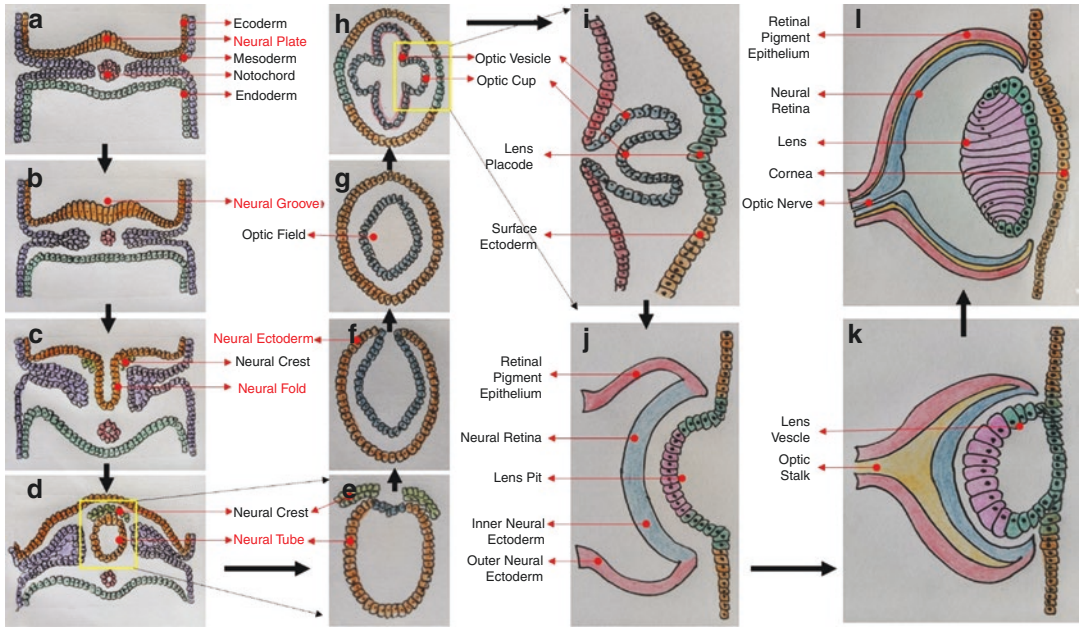

Fig. 24.2 Stages of eye development. Formation of neural tube from neural plate (**a–d**), eye field and optic vesicle from neural tube (**e–h**), optic cup and lens placode (**i**), retina from lens pit (**j**), lens and cornea from surface ectoderm (**k**), and eye (**l**). *Image was redrawn using models from the following sources:* (**a-d**) https://moodle.clsd.k12.pa.us/district_videos/Biology/iText/products/0-13-115540-/ch39/ch39_s4_2_pr.html (**e–h**) http://webvision.umh.es/webvision/develop.html, (**i–k**) Richardson R, Tracey-White D, Webster A and Moosajee M. The zebrafish eye—a paradigm for investigating human ocular genetics. Eye (2017) 31,68–86

The eye field is surrounded by the telencephalic precursors and axial neural epithelial cells that form the hypothalamus [61].

24.4.3 Separation of Eye Field

Separation of the eye field is regulated by the expression of sonic hedgehog (SHH) in the axial neural epithelium upon the influence of Nodal ligands secreted by prechordal axial mesoderm underlying the anterior neural plate. Hedgehog is required to impose hypothalamic fate on the initial medial eye field. Nodal signaling is required for the formation of a prechordal plate; Nodal deficient zebrafish mutants fail to form hypothalamic progenitors. Being deprived of SHH source, Nodal deficient mutants do not separate the eye field, thus exhibiting cyclopia, characterized by the failure of the embryonic prosencephalon to properly divide the orbits of the eye into two cavities [62–65]. SHH mutants also display cyclopia, demonstrating that Hedgehog signaling from the hypothalamus is necessary for eye field separation [66–68]. Post neurulation, SIX3 is required to maintain expression of shh, which in turn maintains six3 expression in the diencephalon, in a positive regulatory loop. Loss of six3 expression in the nascent neural tube results in the loss of hedgehog expression and therefore in failure to separate the eye field [69, 70].

24.4.4 Formation of the Neural Tube and Optic Vesicle

Optical vesicle formation is characterized by the appearance of symmetrical bilateral evagina-

tions from the ventral forebrain (diencephalon), which slowly expand through the mesenchyme toward the surface ectoderm. Evagination of the optic vesicles during the final stages of neural tube formation is the first morphological sign or hallmark of eye morphogenesis (Fig. 24.2h–i). Findings from previous studies (using mouse, fish, and frog models) revealed a coordinated change in cell shape and cellular behavior during the evagination of the optic vesicles [71, 72] partly by a retinal homeodomain transcription factor (Rx/RAX). Further, RAX gene knockout models (mouse (Rx), frog (Rx1), zebrafish (Rx3/chokh), and medaka (eyeless)) display anophthalmia phenotypes like human [73–79], indicating that Rx genes are essential for early eye development. Further, extensive cell movements—the movement of retinal progenitor cells away from the midline and outward—that are integral to the evagination of the optic vesicle are mediated by Rx/RAX through downregulation of NIcam, an Ig-domain cell adhesion molecule. Overexpression of NIcam in Rx3 null mutant resulted in microphthalmia. RAX is suggested to participate in suppressing canonical wnt pathway to prevent the induction of posterior fates of the anterior neural tube and promoting non-canonical wnt signaling pathway to control morphogenetic movement of the ocular cells. Six6 (Optx2), which controls proliferation in the eye field, is also dependent on Rx function [54, 80]. Further, Rx is essential for the expression of other key regulators of early eye formation such as Lhx2, Pax6, Mab2112, Six3/6, Vsx2 to control, directly or indirectly, specification of retinal progenitor cells in the optic vesicle. These findings suggest that Rx regulates the expression of diverse genes that are involved in proliferation and segregation behavior of retinal progenitor cells [81–83] and enables the formation of the optic vesicle.

24.4.5 Formation of the Optic Cup and Lens Placode

As the evaginating optic vesicles contact the mesenchyme and the ectoderm, they form a highly interactive system in which numerous consecutive and frequently reciprocal inductive interactions take place. Establishment of contact between the optic vesicle and surface ectoderm facilitates both the tissues to undergo complex structural changes. The surface ectoderm thickens initially into a "lens placode," which invaginates into a vesicle that eventually closes and separates completely from the surface ectoderm. The concomitant invagination of the optic vesicle results in the formation of double-walled optic cup connected to the diencephalon by the optic stalk (Fig. 24.2j–k). Cyclopia can also occur after optic vesicle evagination. At that stage, SHH induces the optic stalk marker pax2 and represses the retinal marker pax6. The loss of pax2 expression triggers the expansion of pax6 expression medially, inducing retinal fate at the expense of optic stalk fate, leading to fusion of the bilateral optic vesicles [66, 67]. The anterior ventral domain of the optic vesicle may be the driving force for the morphogenesis of the eye and proper specification of ocular tissues. FGF family members expressed in the surface ectoderm appear to induce neural retina formation [reviewed by 51, 84, 85]. In addition, upon contact with the surface ectoderm the prospective neural retina itself expresses FGF8 and FGF9, both of which play a role in defining the boundary between neural retina and RPE [86–89]. Extraocular mesenchyme promotes RPE differentiation, on the other hand, possibly through an activin-like signal [90, 91]. BMP7 expression within the prospective RPE domain helps to maintain the identity of this tissue by antagonizing possible neutralizing effects of FGF [88].

24.4.6 Formation of Retinal Pigment Epithelium and Neural Retina

The dorsoventral patterning of optic vesicle regulation is governed by the bone morphogenetic protein 4 (Bmp4), SHH, and retinoic acid. SHH is also required for central-to-periphery patterning of the optic cup at a later stage [92]. The specification of the neural retina and RPE domains

within the optic vesicle appears to be determined by inductive signals originating in the surface ectoderm and in the mesenchyme, respectively [88–90]. The invagination of the dorsal aspect of the optic vesicle generates an internal layer (the neural retina) and an external layer (the retinal pigment epithelium, RPE), whereas more ventrally the optic vesicle narrows considerably into the "choroid fissure." The fissure closes completely in normal development, forming the optic nerve through which retinal ganglion cell axons grow toward brain; its abnormal persistence is known as "coloboma." Inductive influences from the optic vesicle derivatives influence further development of lens [51, 93, 94]. Neuroepithelial cells of the early optic vesicle co-express Rx, Pax6, Hes1, Otx2, Lhx2, Six3, and Six9, while they are still competent to orginate the optic stalk, neural retina, and RPE [reviewed by 51, 85]. The subsequent specification of these optic vesicle derivatives is accompanied by differential expression of these and other transcription factors: Pax2 and Vax in the prospective optic stalk: Pax6, Rx, Lhx2, and Chx10 (Vsx2) in the prospective neural retina, and Pax6, Otx2, and Mitf in the prospective RPE [51, 84, 85]. Reciprocal transcriptional repression between transcription factors may contribute to establishing boundaries between developing territories (e.g., Pax6 and Pax2 for neural retina and optic stalk, Chx10, and Mitf for neural retina and RPE [87, 95, 96].

The dorsoventral patterning of the optic cup is regulated by a balance between opposing signals originating in the neural tube and/or optic stalk on one hand and in the dorsal region of the optic cup on the other. The earliest known patterning gene is the LIM homeobox transcription factor Lhx2, which is first expressed in the eye field and is required for the expression of Mitf and for retinal determinants in the optic vesicle [54, 97]. Pax6, Hes1, and Lhx2 are necessary for proper growth of the optic vesicle and its transformation into an optic cup. Downregulation of Pax6 in the optic vesicle neuroepithelium affects the survival of optic vesicle cells and the transformation of the optic vesicle into a normal optic cup. Similarly, Lhx2 knockout mice develop optic vesicles, but the optic cup and lens formation fail to occur [reviewed by 51, 98]. The phenotype of Hes1 mutant mice varies from a reduced lens accompanied by a smaller than normal optic cup to the complete absence of the lens with an arrested optic vesicle [99, 100, reviewed by 101]. In the optic cup, interactions between Pax6, Pax2, Vax, and Tbx5 mediate dorsoventral patterning of the neural retina (Fig. 24.3) [reviewed by 51, 96, 102, 103].

24.4.7 RPE Specification

The RPE is required for growth of the eye, it controls proper lamination of the retina, and it regulates differentiation of the photoreceptors [84, 85, 104]. Genetic ablation of the RPE or disruption of RPE specification genes results in microphthalmia, RPE-to-retina transdifferentiation, and coloboma during murine eye development [105–109]. The RPE is specified at the early optic vesicle stage, long before pigmentation becomes obvious. Two key players in RPE specification are the transcription factors Mitf and Otx2 (Fig. 24.3). Mitf is the first gene that is specifically expressed in the presumptive RPE in the optic vesicle [reviewed by 84, 85]. Mitf is a key regulator of pigment cell development in the RPE and neural crest; it transactivates crucial genes for terminal pigment differentiation (e.g., Dct, Tyrp1, and tyrosinase). Otx2 is expressed in the eye field and expression appears to persist until the late optic vesicle stage when it is downregulated in the presumptive retina, like Mitf. Otx2 is required for Mitf expression and transactivates expression of pigment genes in cooperation with Mitf [85, 106, 110].

24.4.8 Neural Retina Specification

The MAP kinase FGF signaling pathway is important for different steps of neural retina development. First, it is essential for patterning of the retina in the distal optic vesicle, and, second, for initiation of retinal neurogenesis. FGF

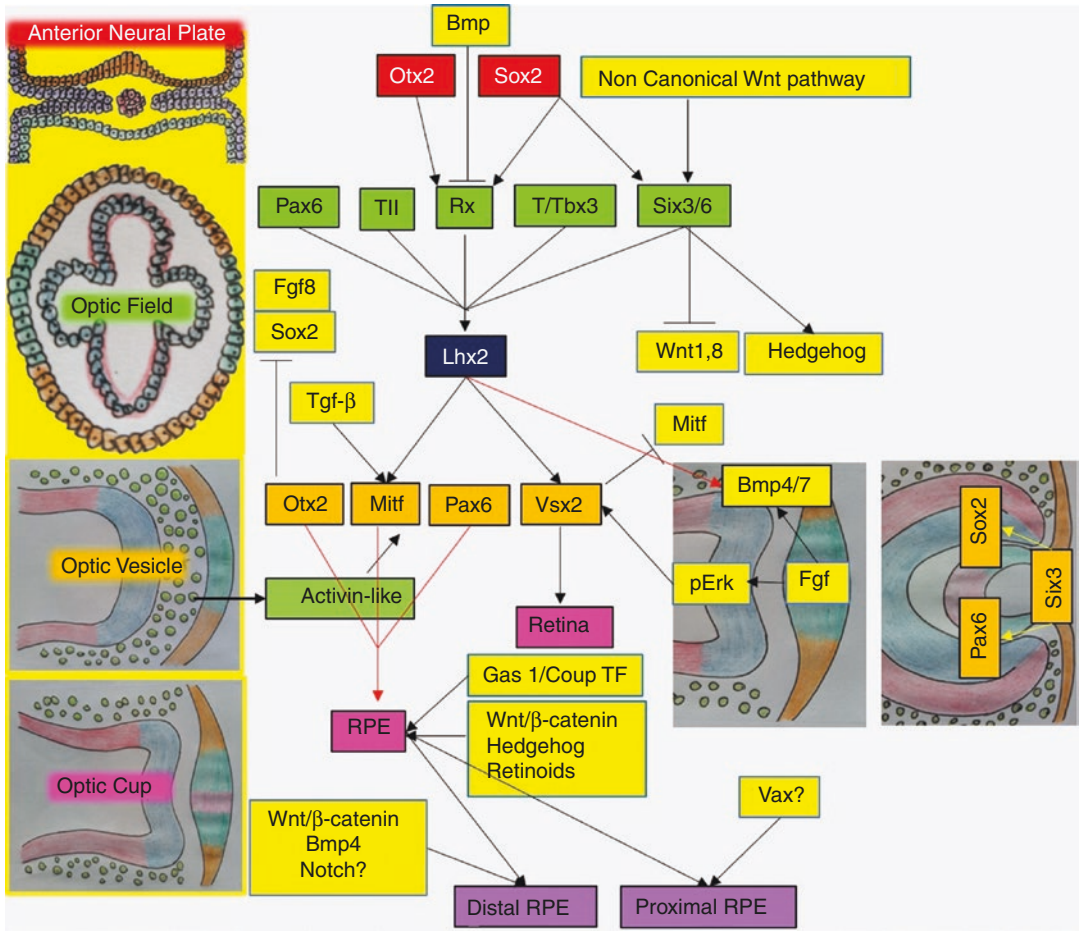

Fig. 24.3 Flowchart depicting complex network and coordinated regulation of signaling molecules and transcription factors in different stages of eye development. The eye field transcription factors Pax6, Rx, Otx2, Six3, as well as Lhx2 are required in the optic vesicle to respond to inducing signals. During RPE specification in mouse (early optic vesicle), the extraocular mesenchyme, possibly by producing an activin-like factor, induces Mitf expression in the entire optic vesicle. However, in mouse and chick (late optic vesicle), activation of ERK, potentially through FGF secreted from the lens (surface) ectoderm, induces/maintains Vsx2 and Sox2 expression in the distal optic vesicle to promote retina development, which requires Vsx2-mediated suppression of Mitf. Invagination of lens placode requires correct specification of the lens (surface) ectoderm that is dependent on Six3-mediated maintenance and activation of Pax6 and Sox2, respectively. In the distal optic vesicle, BMP4 and BMP7 expression is crucial for specification of the lens ectoderm and for optic vesicle invagination. BMP expression requires activation by Lhx2 and FGF signaling. Several signaling pathways regulate maintenance of cell fate in the presumptive RPE in the optic cup. *Reproduced with permission (Licence No. 4800211090836) from Elsevier and Copyright Clearance Centre from* Sabine Fuhrmann, ChangJiang Zou, and Edward M. Levine. Retinal pigment epithelium development, plasticity, and tissue homeostasis. Exp Eye Res. 2014 Jun; 0: 141–150. doi: [10.1016/j.exer.2013.09.003] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4087157/ [278]

ligands and receptors are abundantly expressed in ocular and extraocular tissues, and specifically, FGF1 and FGF2 show strong expression in lens ectoderm [88, 111, 112]. FGF derived from the lens ectoderm is necessary to maintain the surface ectoderm [113]. BMP signaling may also participate in early steps of retina development and is supported by the finding that BMP7 null mice display varying incidents of microphthalmia or anophthalmia, depending on the genetic background. In anophthalmic BMP null mice, the expression of retina-specific genes is downregu-

lated in the optic cup, with concomitant ectopic expression of RPE genes such as Mitf [114].

24.4.9 Formation of Lens and Other Ocular Structures

The distal portion of the optic vesicle contacts the overlying surface ectoderm, resulting in the specification of the lens ectoderm (pre-placodal stage). This interaction leads to invagination of the lens placode and distal optic vesicle resulting in the formation of a bilayered optic cup. Invagination of lens placode requires correct specification of the lens ectoderm that is dependent on Six3-mediated maintenance and activation of Pax6 and Sox2, respectively. Recent studies revealed that Six3 is expressed in the surface ectoderm before Pax6, and without Six3, Pax6 is downregulated and Sox2 never expressed [115]. It demonstrates that Six3 directly regulates the expression of Pax6 and Sox2 and indicates that Six3, Pax6, and Sox2 act in a complex regulatory network to regulate each other during lens induction and specification [115, reviewed by 116, 117]. FGF and BMP signaling may be also required for lens induction. In the distal optic vesicle, BMP4 and BMP7 expression is crucial for the specification of the lens ectoderm and for optic vesicle invagination. BMP expression requires activation by Lhx2 and FGF signaling. The lens vesicle eventually separates from the surface ectoderm, which gives rise to the corneal epithelium and differentiates into the mature lens. The lens also influences the formation of the iris and ciliary body, specialized structures at the peripheral margin of the optic cup [118].

Thus, the development of the vertebrate eye is formed through the coordinated interactions between the germinal layers (neuroepithelium, surface ectoderm, and extraocular mesenchyme) and the intricate network of signaling molecules and gene regulatory factors [119, 120]. Majority of signaling and transcription factor genes show restricted spatiotemporal expression; however, a few of them reappear at different stages of eye development, controlling different developmental events (Fig. 24.3 and Table 24.2). As these factors reappear at different events, tight genetic and epigenetic mechanisms might play a crucial role in normal eye development. Owing to multiple embryological derivations and interactions of several regulatory factors, disturbance at any of the developmental stages or in the regulatory factors consequently would result in variable phenotypes of congenital ocular anomalies such as anophthalmia and microphthalmia that could potentially result in blinding conditions.

Table 24.2 Key signaling molecules and transcription factors in eye development

Events during eye development	Signaling molecules	Transcription factors
Specification of the eye field	FGFs, Wnts, BMPs, Cyclops, SHH	Rx, Pax6, Six3, Lhx2, Six6/Optx2, ET, tII, HesI, Otx2
Formation and patterning of Optic vesicle	SHH, FGFs, Activin, BMP7, retinoic acid	Rx, Pax6, Tii, Lhx2, Vsx2/ Chx10, Otx2, Mitf, Pax2, Vax, BF1/FoxgI, BF2/ Foxd2
Formation of optic cup by invagination of the optic vesicle	Retinoic acid	Pax6, Lhx2, HesI
Patterning of the optic cup	Nodal, FGFs, SHH, BMPs, RA, Ventroptin, Follistatin, Chordin, Noggin, DAN	Pax6, Pax2, Vax, Tbx5, Xbr1, BF1/ Foxg1, BF2/ Foxd2, SOHo1, GH6

BMPs bone morphogenetic proteins, *DAN* DAN domain family members, *FGFs* fibroblast growth factors, *SHH* sonic hedgehog, *Wnts* members of the Wnt family
Source: Ref: 101. Adler R and Canto-Soler MV (2007). Molecular mechanisms of optic vesicle development: Complexities, ambiguities and controversies. Developmental Biol. 305: 1–13

24.5 Genetics of Microphthalmia: Indian and Global Perspectives

Although much information on eye development has come from animal models such as mouse, frog, chick, and zebrafish, the molecular signaling factors and other genes involved in the regulation have more similarity with humans. At least eighty-two genes are currently known to be

associated with microphthalmia. Among them, transcription factors represent the largest group (ATOH7, SOX2, OTX2, FOXE3, PAX6, VSX2, PITX3, RAX, SIX6, SALL2), which is followed by TGFB/BMP signaling molecules (BMP4, BMP7, GDF6, GDF3), retinoic acid pathway genes (ALDH1A3, STRA6, RARB), and other genes with known or unknown functions (SHH, ABCB6, MAB21L2, C120rf37, TENM3 (ODZ3), PXDN, YAPI, HMGB3, and CRIM1). A brief account of the functions and role of some of the important TGFB/BMP signaling factors, transcription factors, and retinoic acid pathway genes is given in Table 24.3.

Table 24.3 Functions of some of the key signaling molecules and transcription factors

Protein family	Genes	Functions and phenotypes
TGFB/BMP family of secretory signaling molecules	BMP4	• Plays an important role in the embryonic development and responsible for the dorsoventral patterning of the optic vesicle [121, 122] • Mice with $Bmp4^{-/-}$ homozygotes die during early embryogenesis, while the $Bmp4^{+/-}$ heterozygotes show microphthalmia, anterior segment dysgenesis, failure of lens induction, and retinal and optic nerve aplasia [123–125] • Excessive Bmp4 signaling is also known to decrease eye growth leading to small and misshapen eyes
	GDF3	• Plays essential role in ocular and skeletal development [23] • $Gdf3^{-/-}$ mice do not display any ocular defects [126]; however, morpholino knockdown of dvr1, an ortholog of gdf1/3, leads to coloboma, decreased eye size, tail curvature, and reduced tail length in a few embryos [127] • Mutations in GDF3 are associated with microphthalmia, coloboma, and skeletal defects (vertebral fusion, scoliosis, rudimentary 12th rib) [127].
	GDF6	• Plays a crucial role in regulating the patterning during development [128] • Mutations in GDF6 are associated with a wide range of ocular phenotypes including microphthalmia, anophthalmia, or coloboma (MAC) developmental spectrum [129]. The loss-of-function mutation in GDF6 gene was initially associated with skeletal abnormalities and consequently with ocular defects such as microphthalmia and coloboma [129, 130]
Retinoic acid pathway	ALDH1A3	• Oxidizes retinaldehyde to retinoic acid [23] • Knockdown of aldh1a3 in zebrafish via morpholino leads to microphthalmia with late closure of optic fissure and coloboma in few larvae [131] • Mutation in ALDH1A3 is associated with bilateral anophthalmia/microphthalmia, and coloboma [23]
	RARB	• Involved in eye development through vitamin A pathway [132] • Mutations in RARB are known to be associated with microphthalmia, anophthalmia, and coloboma through dominant and recessive mode of inheritance [132]
	STRA6	• Plays an important role in vitamin A homeostasis of peripheral tissues [133] • Mutation in STRA6 is known to be associated with PDAC (pulmonary aplasia/agenesis/hypoplasia, diaphragmatic eventration/hernia, anophthalmia/microphthalmia, and cardiac defect) syndrome [134, 135]
Basic helix-loop-helix family of transcription factors	ATOH7	• This intronless gene has similarity with Drosophila atonal gene that regulates photoreceptor development [136] • Plays a crucial role in the retinal ganglion cell and optic nerve formation Mutation in ATOH7 has been described to be associated with non-syndromic congenital retinal non-attachment [137], vitreoretinal dysplasia, optic nerve hypoplasia, persistent fetal vasculature, microphthalmia, microcornea, corneal opacity, congenital cataracts, and nystagmus [138].
Basic helix-loop-helix family of transcription factors	MITF	• Plays an essential role in the closure of optic fissure, development, and homeostasis of bone. Mutation in MITF is associated with COMMAD (coloboma, osteopetrosis, microphthalmia, macrocephaly, albinism, and deafness [139]

Table 24.3 (continued)

Protein family	Genes	Functions and phenotypes
Zinc-finger	SALL2	• Plays pivotal role in optic fissure closure during eye morphogenesis • Sall2 deficient mouse embryos show delayed apposition of the optic fissure margins and anterior retinal coloboma after birth. Mutation in SALL2 is associated with recessive ocular coloboma in humans and mice [140]
Winged helix-forkhead family member	FOXE3	• Plays a crucial role in the development of lens placode [141] • Primarily expressed in the developing brain and inside the lens placode; later becomes restricted to the anterior lens epithelium when fiber cell differentiation begins [142]. • Foxe3 maps to a chromosomal region consisting of the dysgenetic lens (dyl) mutation (Brownell et al., 2000). Homozygous dyl mice show several defects in lens formation. They also display altered patterns of crystalline expression signifying deregulation in lens differentiation • Dyl mouse has an autosomal recessive mutant phenotype characterized by small eyes, iris adhesions, corneal opacities, cataracts, and persistent attachment of lens and cornea [143]
Bicoid-type homeodomain	OTX2	• Play a crucial role in the specification of the eye, predominantly the RPE [54, 106, 110, 144, 145] • Otx2 null embryos show a severe head phenotype involving lack of anterior neuroectoderm and deformities in the body plan [146, 147]. • The $Otx2^{+/-}$ mice might be normal or possess developmental eye disorders such as anterior segment anomalies, microphthalmia, anophthalmia, or head deformities [144, 146, 147]
Paired domain gene	PAX6	• It is crucial for the activation of genes involved in the formation of the eye, central nervous system, and pancreas [148] • In zebrafish, homozygous pax6b mutants have thick cornea, iris hypoplasia, small lens, and shallow anterior chamber [149] • Mutation in PAX6 is associated with aniridia [150], congenital cataract [151], glaucoma [152], nystagmus [153], microphthalmia [154], microcornea [155], iris coloboma [154], iris, and foveal hypoplasia [156]
HMG-box domain	SOX2	• Plays crucial role in the normal development of different ocular tissues [123, 157–159] • Mutation in *SOX2* is a common cause of anophthalmia, microphthalmia, and coloboma [160, 161]
Homeodomain	PITX3	• Plays a crucial role in retinal and lens development [162, 163] • In zebrafish, pitx3 knockdown via morpholino results in small eyes with retinal defects, lens degeneration, jaw abnormalities, and misshapen heads [162, 163] • Mutation in PITX3 is associated with congenital cataract, bilateral microphthalmia, and neurodevelopmental abnormalities [164]
	RAX	• RX/RAX is a well-conserved essential homeobox protein initially expressed in the eye field and then in the budding bilateral optic vesicles • It plays an essential role in retinal cell fate determination and regulation of stem cell proliferation [50] • RX/RAX misexpression induces ectopic eye formation in Xenopus • In fish (zebrafish and medaka) and mouse, homozygous loss-of-function mutants do not exhibit any eye structure, demonstrating that this protein is crucial for eye formation in vertebrates [76, 77, 83] • In mice, loss of Rax function leads to anophthalmia and brain defects [77] • In humans, *RAX* gene mutations are linked to anophthalmia, microphthalmia, and coloboma [79, 165]
	SIX3	• Plays an important role in mammalian lens induction and specification and optic nerve development [115, 166] • In mouse, conditional deletion of Six3 in the presumptive lens ectoderm results in defective lens formation [115] • In zebrafish, decreased Six3 function is associated with optic nerve hypoplasia [166]

(continued)

Table 24.3 (continued)

Protein family	Genes	Functions and phenotypes
	SIX6	• Plays a crucial role in the early stages of pattern formation in the eye disc [167] • In mice, disruption of Six6 gene has been shown to cause pituitary and retinal defects usually with the absence of optic chiasma and optic nerve that resembles the human phenotype [168] • In humans, SIX6 mutations are associated with microphthalmia, anophthalmia, and coloboma [169]
	VSX2	• Expressed in the retina of human [170], mouse [171], and the zebrafish embryos [172, 173] • VSX2 mutations are associated with autosomal recessive anophthalmia/microphthalmia with or without iris coloboma and other ocular disorders. In most of the cases, the ocular defects are isolated; however, in a few cases, extraocular features have also been described consisting of hormone deficiency and learning difficulties [174]
Transcription factor	LHX2	• Plays an important role in the neural differentiation of human stem cells [175]. It is expressed in the posterior pituitary, eye, and liver during early eye development • Lhx2 (−/−) mice exhibit lack of posterior pituitary and intermediate lobes, anophthalmia, malformation of the anterior lobe, and die from anemia [98, 176, 177] • In humans, mutation in Lhx2 is known to be associated with anophthalmia [178].
Transcription factor	VAX	• Vax1 and Vax2 play an important role during eye development and the closure of choroid fissure in mice and zebrafish [179] • In humans, mutation in VAX1 causes microphthalmia, orofacial clefting, and corpus callosum agenesis [179].

Ample reports on the genetics of microphthalmia and associated ocular and systemic conditions have been published in recent years with the advent of high-throughput next-generation sequencing tools such as whole genome sequencing (WGS), whole exome sequencing (WES), and targeted exome sequencing (TES). As a result of this, several novel genes (including CRYBA4, CRYBA2, BFSP1, VIM, HSF4, and EZR) and novel mutations in genes RAX, SOX2, OTX2, CHX10 (VSX2), PAX6, FOXE3, ABCB6, SHH, and NDP have been reported [197]. As new genes for microphthalmia are identified in the genomic era, the number of syndromes associated with microphthalmia has greatly expanded (Table 24.4). Covering all the candidate genes and their genetic variations associated with microphthalmia is beyond our scope; therefore, this chapter essentially deals with genetic mutations of a few candidate genes of signaling (BMP4), and transcription factors (FOXE3, OTX2, PITX3, RAX, SIX6, SOX2, and VSX2) responsible for normal eye development and microphthalmia.

24.5.1 Genetic Studies on Microphthalmia: Global Perspectives

24.5.1.1 BMP4 Gene and Microphthalmia

BMP4, mapped to chromosome 14q22-q23, is a member of the *BMP* family and transforming growth factor-β1 (TGF-β1) superfamily of secretory signaling [216, 217]. It is composed of four exons (although only exons 3 and 4 are translated); the protein is 408 amino acids long and consists of a TGF-β1 propeptide domain and a TGF-β domain that forms an active dimer [218]. The finding of expression of BMP4 in developing human optic vesicle, retina, lens, pituitary region, and digits strongly supported BMP4 as a causative gene for anophthalmia-microphthalmia

Table 24.4 Genes associated with isolated and syndromic microphthalmia

Microphthalmia type	ID	Inheritance	Phenotype/Gene/locus OMIM no.	Chr. Loci	Gene(s)/Locus	References
Microphthalmia isolated 1 Anophthalmia and microphthalmia	MCOP1	AR	251600	14q32	MCOP1	[39]
Microphthalmia isolated 2 Clinical anophthalmia associated with cataract	MCOP2	AR	142993 610092	14q24.3	VSX2	[242– 244]
Microphthalmia isolated 3 Associated with sclerocornea, developmental delays	MCOP3	AR	601881 611038	18q21.32	RAX	[79, 165]
Microphthalmia isolated 4	MCOP4	AD	601147 613094	8q22.1	GDF6	[129]
Microphthalmia isolated 5 Associated with microphthalmia, posterior, with retinitis pigmentosa, foveoschisis, and optic disc drusen	MCOP5	AR	611040 606227	11q22.3	MFRP	[180]
Microphthalmia isolated 6 Anophthalmia associated with retinal dystrophy, sclerocornea, brain, digital (poly/syndactyly), and pituitary anomalies, malformed ears, micrognathia, and abnormal external genitalia, orofacial cleft 11, and learning disabilities	MCOP6	AR	613517 613858	2q37.1	PRSS56	[181, 182]
Microphthalmia isolated 7	MCOP7	AD	613704 606522	12p13.31	GDF3	[127]
Microphthalmia isolated 8	MCOP8	AR	615113 600463	15q26.3	ALDH1A3	[183]
Microphthalmia isolated with Coloboma 1	MCOPCB1	–	300345	XL	MCOPCB1	[184]
Microphthalmia isolated with Coloboma 2	MCOPCB2		605738	15q12-q15	MCOPCB2	[185, 186]
Microphthalmia isolated with Coloboma 3	MCOPCB3	AR	610092 142993	14q24.3	VSX2	[187–189]

(continued)

Table 24.4 (continued)

Microphthalmia type	ID	Inheritance	Phenotype/Gene/locus OMIM no.	Chr. Loci	Gene(s)/Locus	References
Microphthalmia isolated with Coloboma 4	MCOPCB4	–	251505	Not mapped	MCOPCB4	[190, 191]
Microphthalmia isolated with coloboma 5	MCOPCB5	AD	611638 600725	7q36.3	SHH	[192]
Microphthalmia isolated with coloboma 6	MCOPCB6	AD	613703	12p13.31	GDF3	[127]
Microphthalmia isolated with coloboma 6, digenic	MCOPCB6	AD	613703	8q22.1	GDF6	[127]
Microphthalmia isolated with coloboma 7	MCOPCB7	AD	614497 605452	2q35	ABCB6	[193]
Microphthalmia isolated with coloboma 8	MCOPCB8	AR	601186 610745	15q24.1	STRA6	[42, 134, 194]
Microphthalmia isolated with coloboma 9	MCOPCB9	AR	615145 610083	4q34.3-q35.1	ODZ3, TENM3	[35, 195]
Microphthalmia isolated with coloboma 10	MCOPCB10	AD	616428 180250	10q23.33	RBP4	[196]
Microphthalmia/coloboma and skeletal dysplasia syndrome	MCOPS14	AD/AR	615877 604357	4q31.3	MAB21L2	[197, 198]
Microphthalmia syndromic 1 Associated with Lenz microphthalmia syndrome, lens dysplasia	MCOPS1	XL	309800	Xq28	NAA10	[199]
Microphthalmia syndromic 2 Associated with oculo-facio-cardio-dental syndrome (OFCD) in females, cataracts, tooth anomaly, radiculomegaly, toe anomalies, and septal heart defects. Lenz microphthalmia syndrome in males only	MCOPS2	XLD	300166 300485	Xp11.4	BCOR	[200, 201]

Microphthalmia syndromic 3 Associated with or without optic nerve defects and defects of the optic chiasm and optic tract, failure to thrive, genital abnormalities, developmental delay, ataxic gait, atypical seizures, motor disability, brain disorders, sensorineural hearing loss, neurocognitive delays, esophageal atresia with or without tracheoesophageal fistula, and pituitary hypoplasia	MCOPS3	AD	206900 184429	3q26.33	SOX2	[202–204]
Microphthalmia syndromic 4 Associated with microphthalmia with ankyloblepharon and mental retardation	MCOPS4	XLR	301590	Xq27-q28	MCOPS4	[205]
Microphthalmia syndromic 5 Associated with anterior segment dysgenesis, retinal dystrophy with or without pituitary anomalies/ dysfunction, optic nerve hypoplasia/ dysplasia, brain abnormalities, developmental delays, and autistic features	MCOPS5	AR	610125 600037	14q22.3	OTX2	[18]
Microphthalmia syndromic 6 Associated with brain, digits, pituitary, and genital abnormalities	MCOPS6	AD	607932 112262	14q22.2	BMP4	[206, 207]
Microphthalmia syndromic 8 Associated with microphthalmia, microcephaly, ectrodactyly of lower limbs, and prognathism	MCOPS8	?AD	601349	6q21	MCOPS8	[208, 209]
Microphthalmia syndromic 9 Linked to the pulmonary hypoplasia-diaphragmatic hernia-anophthalmia-cardiac defect which is characterized by pulmonary hypoplasia, diaphragmatic defects, bilateral anophthalmia, and cardiac disorders	MCOPS9	AR	601186	15q24.1	STRA6	[42, 134, 194]

(continued)

Table 24.4 (continued)

Microphthalmia type	ID	Inheritance	Phenotype/Gene/locus OMIM no.	Chr. Loci	Gene(s)/Locus	References
Microphthalmia syndromic 10 Associated with microphthalmia and brain atrophy	MCOPS10	–	611222	Not mapped	MCOPS10	[210]
Microphthalmia syndromic 11 Consists of microphthalmia along with cleft lip and palate, corpus callosum agenesis	MCOPS11	AR	614402 604294	10q25.3	VAX1	[179]
Microphthalmia syndromic 12 Involves microphthalmia with or without a diaphragmatic hernia, developmental delays, bicornuate uterus, intestinal malrotation, hypotonia, pulmonary hypoplasia, and/or cardiac anomalies	MCOPS12	AD and AR	615524 180220	3p24.2	RARB	[211]
Microphthalmia syndromic 13 Characterized by microphthalmia along with coloboma, short stature, and psychomotor retardation	MCOPS13	XL	300915 300193	Xq28	HMGB3	[212]
Optic nerve hypoplasia and abnormalities of the central nervous system	MCOPS3	AD	206900 184429	3q26.33	SOX2	[213]
Retinal dystrophy, early onset, with or without pituitary dysfunction	MCOPS5	AD	610125 600037	14q22.3	OTX2	[214]
Linear skin defects with multiple congenital anomalies 1	LSDMCA1	XLD	309801 300056	Xp22.2	HCCS	[215]

Source: https://omim.org/

with pituitary abnormalities and digit anomalies. The first case of BMP4 gene deletion [14q22-23] was reported in three patients with anophthalmia, developmental delay, and structural brain malformations with syndactyly, brachydactyly, pituitary defects, and genitourinary anomalies [206, 219]. Bakrania et al.[206] analyzed 215 sporadic cases having microphthalmia. They reported deletion of BMP4-OTX2 gene region [46XX, del(14)(q22.3q23.2) and 46XY,del(14)(q22.3q23.1)] in two cases with bilateral anophthalmia-microphthalmia, a frameshift mutation in a family with anophthalmia-microphthalmia, retinal dystrophy, myopia, poly- and/or syndactyly, and brain anomalies, and a missense mutation in an individual with anophthalmia-microphthalmia and brain anomalies. Reis et al. (2011) [207] analyzed the BMP4 coding region in 133 patients with various ocular conditions and reported a heterozygous deletion of 158 kb in the BMP4 gene region, a nonsense mutation (p.Arg198Ter), and a frameshift mutation (p.Glu58ArgfsTer17), respectively, in 3 probands with syndromic microphthalmia. The proband carrying a nonsense mutation has conditions of anophthalmia, microphthalmia with sclerocornea, right-sided diaphragmatic hernia, and hydrocephalus, and the two-half siblings harboring frameshift mutation had the conditions of anophthalmia/microphthalmia and discordant developmental delay/postaxial polydactyly. The affected sister of the proband with the frameshift mutation carried an additional missense mutation (p.His121Arg) along with frameshift mutation.

24.5.1.2 FOXE3 Gene and Microphthalmia

FOXE3 maps to chromosome 1p33. This intronless gene belongs to the forkhead family of transcription factors, which is characterized by a distinct forkhead domain. In humans, a mutation in *FOXE3* was first described by Semina and coworkers [220] in a patient with posterior embryotoxon, cataract, and myopia. FOXE3 gene mutations were also reported in non-syndromic microphthalmia conditions. Ali and coworkers [221] reported nonsense and a missense mutation in two consanguineous families from Pakistan and Mexico, respectively, having bilateral non-syndromic microphthalmia with sclerocornea. The patients with sclerocornea also had microphthalmia, and few others had aphakia and optic disc coloboma. Garcia-Montalvo et al. [222] reported a homozygous deleterious mutation in a patient with bilateral microphthalmia and sclerocornea. In contrast, Sano and coworker [223] did not find any mutation in the case of a 2-month-old patient with congenital primary aphakia and associated syndromes such as microphthalmia, corneal opacity, and dysplasia of the anterior segment.

24.5.1.3 OTX2 Gene and Microphthalmia

OTX2 gene maps to chromosome 14q22.3 and contains an extremely conserved bicoid-type homeodomain. It consists of five exons out of which only three are coding. OTX2 gene mutations are associated with ocular, developmental, pituitary hormone deficiency, and other systemic disorders [224, 225]. In 333 patients with ocular malformations, Ragge et al. [226] reported heterozygous mutations in the OTX2 gene in 11 affected individuals with syndromic microphthalmia. Wyatt et al. [227] analyzed 165 patients with clinical anophthalmia, microphthalmia, and/or coloboma and identified 2 heterozygous whole gene deletions, involving OTX2 and several other genes, and 2 nonsense and 2 frameshift mutations in 8 patients. Tajima et al. [228] identified a de novo heterozygous frameshift mutation in the OTX2 gene in a 6-year-old Japanese patient with bilateral clinical anophthalmia, short stature, and combined pituitary hormone deficiency. Dateki et al. [224] identified 3 heterozygous truncation mutations and a microdeletion in the OTX2 gene. Chassaing et al. [225] reported a heterozygous 1-bp deletion (c.316delC) in the OTX2 gene in a large 4-generation French family with 17 affected individuals with microphthalmia and clinical anophthalmia. Patat et al. [229] identified a heterozygous nonsense mutation (p.Arg97Ter) in the OTX2 gene in a mother with unilateral severe microphthalmia and her male fetus with agnathia–otocephaly complex. Gerth-Kahlert et al.[230] reported three OTX2

mutations in patients with bilateral anophthalmia (p.Glu79Serfs*30), unilateral anophthalmia (p. Lys92Asnfs*11) and clinical anophthalmia, microphthalmia, microcornea, and retinal coloboma (p.Gln83His). Although OTX2 gene mutations are associated with a spectrum of disorders, the loss-of-function mutations do not sufficiently explain the complex anatomic defects in patients with otocephaly/dysgnathia [224, 225, 231, 232], suggesting the requirement for a second genetic hit.

24.5.1.4 PITX3 Gene and Microphthalmia

PITX3 gene is mapped to chromosome 10q24.32 and belongs to RIEG/PITX homeobox gene family. It consists of 4 exons. Semina et al. [233] were the first to report PITX3 gene mutations in ocular disorders such as anterior segment mesenchymal dysgenesis (ASMD) and congenital cataract. They screened mutations in the PITX3 gene in individuals with various eye anomalies and identified a 17bp insertion that results in a frameshift in one patient and a missense mutation that results in substitution of asparagines by serine in a patient with congenital cataract. Summers et al. [234] identified 17bp duplication in PITX3 gene, previously reported by Withers et al. [235], in large Australian kindred having anterior segment abnormalities, including Peters anomaly with corneal clouding, iridolenticular corneal adhesions, displaced Schwalbe line, and cataract. Berry et al. [236] also identified this 17-bp duplication in 4 large families (3 of English descent and 1 of Chinese descent) with autosomal dominant posterior polar cataract and a heterozygous 1 bp deletion (c.650delG) in a family of Hispanic descent with posterior polar cataract. They noted that all 50 affected individuals had cataract, but only 5 individuals, from 2 of the 5 families, had ASMD in addition to cataract. In 1 family, only 1 member had ASMD, and her affected daughter, who had the same 17 bp duplication, had only cataract. Therefore, it was suggested that cataract, rather than ASMD, may be the major feature of PITX3 mutations, and that there may be another gene causing ASMD. Bidinost et al. [164] identified the 1 bp deletion (c.650delG) mutation in a heterozygous state, previously reported [236], in 26-member 3-generation Lebanese family with posterior polar cataract. In addition, 2 affected brothers from a consanguineous mating in this family were homozygous for the deletion and had posterior polar cataract, severe microphthalmia, and neurodevelopmental abnormalities.

24.5.1.5 RAX Gene and Microphthalmia

RAX is a homeobox-containing transcription factor that plays a very important role in vertebrate eye development. It maps to chromosome 18q21.32 and consists of three exons. Voronina et al. [79] identified a compound heterozygous mutation consisting of a truncated allele p.Gln147Ter and a missense mutation p.Arg192Gln in a patient with clinical anophthalmia and/or microphthalmia and sclerocornea. Lequeux et al. [165] reported a compound heterozygous 1-bp deletion mutation (c.664delT) and a nonsense mutation (p.Tyr303X) in the RAX gene in a 2-year-old Algerian girl with bilateral clinical anophthalmia. London et al. [237] screened 29 microphthalmia-anophthalmia-coloboma (MAC) cases for a mutation in RAX gene and identified a heterozygous mutation (p.Arg66Thr) in a patient with coloboma. Gonzalez-Rodriguez et al. [238] screened 50 unrelated MAC cases and identified two RAX mutations; the first mutation p.Thr50ProTer was identified in a patient with microphthalmia, septum pellucidum, cortical atrophy, and optic nerve atrophy, and the second mutation (p.Arg110GlyTer) was identified in an anophthalmia patient with hydrocephalus and congenital hip dislocation.

24.5.1.6 SIX6 Gene and Microphthalmia

SIX6 is a member of the SIX/sine oculis family of homeobox genes, and it maps to 14q22.3q23. It consists of 2 exons, and it is expressed in the developing and adult retina, optic nerve, hypothalamus, and the pituitary regions [239, 240]. In an individual with bilateral anophthalmia and pituitary disorders, Bennett et al. [241] reported a deletion including the SIX6 gene suggesting SIX6 haploinsufficiency. Following this, Gallardo

et al.[242] screened European pedigrees with syndromic and non-syndromic sporadic cases of anophthalmia/microphthalmia for a mutation in SIX6 gene. They identified three SNPs IVS-185A>G, p.Leu7Leu, and p.Asp141His that showed no association with A/M. They also reported a heterozygous variation p.Thr165Ala in the exon 1 of SIX6 gene in a patient with bilateral congenital asymmetric microphthalmia, cataract, nystagmus, and syndactyly of toes. This variation was also present in the unaffected father and absent in 160 chromosomes from the healthy normal controls. In another study, Aijaz et al. [169] reported an absence of a SIX6 mutation in 173 individuals with microphthalmia, anophthalmia, and coloboma and suggested that there is no solid evidence that mutation in SIX6 alone could lead to microphthalmia, anophthalmia, and coloboma.

24.5.1.7 SOX2 Gene and Microphthalmia

SOX2 maps to chromosome 3q26.33. This intronless gene encodes a member of the SRY-related HMG-box (SOX) family of transcription factors involved in the regulation of embryonic development and in the determination of cell fate. SOX2 represents a high mobility group (HMG) DNA-binding domain containing transcription factor that plays an essential role in the normal development of the vertebrates. Its expression commences early during eye development and is crucial for normal development of different ocular tissues [123, 157–159]. SOX2 consists of three functional domains: the high mobility group (HMG) domain which is necessary for DNA binding, activation or repression domain, and a partner-factor interaction domain. Mutation in SOX2 is a common cause of anophthalmia/microphthalmia. Majority of the SOX2 coding mutations are de novo insertions/deletions leading to premature truncation of the normal protein. Until now, 58 different SOX2 mutations have been reported. Fantes et al. [243] identified a submicroscopic deletion consisting of SOX2 at 3q breakpoint in a child with t(3,11)(q26.3;p11.2) linked to bilateral anophthalmia. Consequently, mutation analysis of SOX2 revealed a de novo truncating SOX2 mutation in 11% of the anophthalmia cases. Chassaing et al.[244] identified 18 heterozygous SOX2 mutations (5 SOX2 deletions, p.Asn33GlyfsX66, p.Trp51Arg, p.Arg53HisfsX37, p.His67ProfsX35 (2), p.Arg74Pro, p.Trp79Ser, p.Leu82CysfsX20, p.Thr85ThrfsX17, p.Glu104X, p.Tyr160X, p.Tyr171, p.Tyr200SerfsX2), in 18 out of the 150 anophthalmia/microphthalmia (A/M) cases screened. They identified eight small intragenic deletions/duplications and three missense and two nonsense mutations in the conserved amino acid region located in the DNA-binding domain. Recently, Ammar et al. [245] identified a previously reported heterozygous nonsense mutation p.Tyr160X in the SOX2 gene in a patient with A/M with craniofacial dysmorphism.

24.5.1.8 VSX2 (Visual System Homeobox 2) Gene and Microphthalmia

VSX2 is a homeodomain-containing transcription factor which was formerly known as *CHX10*. It maps to chromosome 14q24.3and consists of 5 exons. It is expressed in the retina of human [170], mouse [171], and the zebrafish embryos [172, 173], and its deficiency leads to microphthalmia and other associated ocular disorders [170–172] indicating the evolutionarily conserved function of VSX2 gene. VSX2 mutations are associated with autosomal recessive anophthalmia/microphthalmia (A/M) with or without iris coloboma and other ocular disorders. In most of the cases, the ocular defects are isolated; however, in few cases, extraocular features have also been described consisting of hormone deficiency and learning difficulties [174]. Truslove [246] described a null mutation in the Vsx2 gene in mice with microphthalmia phenotype. Later, FerdaPercin et al. [170] reported homozygous mutations (p.R200Q and p.R200P, respectively) in two families with non-syndromic microphthalmia, cataracts, and severe iris abnormalities. In 2002, Morrison et al. [247] reported screening of 150 patients with microphthalmia, anophthalmia, and coloboma from Scotland and found no VSX2 mutation in those patients. Gonzalez-Rodriguez et al. [238] also reported a lack of VSX2 mutation in 50 unrelated microphthalmia, anophthalmia, and coloboma cases from

Mexico. These reports highlight that the genes other than VSX2 might play a causative role in microphthalmia.

24.5.2 Genetic Studies on Microphthalmia: Indian Perspectives

Despite having advanced technologies for genetic testing and mutation screening, reports on genetic studies of microphthalmia in the Indian subcontinent are still in their infancy. Although a few institutes have attempted to understand the genetic causes of microphthalmia in India, a gap in identifying novel candidate genes of microphthalmia continues to exist. The reason could be wide, but the rarity of microphthalmos cases with a strong family history comes to the front line. A study conducted by our group [248] involving sporadic cases of microphthalmia with other noticeable ocular disorders in the western region of India revealed a novel gene and a few novel genetic variations of known candidate genes. The study comprised 319 subjects from western India out of which 267 were controls, and 52 were with symptoms of microphthalmia. Out of 52 A/M cases, 10 (19.23%) had bilateral anophthalmia, 17 (32.69%) had isolated bilateral microphthalmia, 19 (36.54%) had syndromic bilateral microphthalmia, and 6 (11.54%) had one eye anophthalmia and another eye with microphthalmia. The syndromic cases of microphthalmia included eye phenotypes such as congenital cataract, leukocoria, iris, and chorioretinal coloboma, microcornea, and non-ocular phenotypes such as tooth anomaly, developmental delay, and cardiac defect. Out of 52 A/M cases, only two cases had a family history of microphthalmia.

Mutation screening of all cases was performed using Sanger's sequencing technology for 10 candidate genes comprising TGFB/BMP family of signaling factors (BMP4), transcription factors (FOXE3, OTX2, PITX3, RAX, SIX6, SOX2, VSX2), and gap junctions (GJA3 and GJA8). For the first time, we reported the involvement of novel candidate genes, GJA3 and GJA8, and a few pathological mutations (Table 24.5) in microphthalmia from the Indian subcontinent in addition to 5 novel nucleotide variations and 13 reported polymorphisms in known candidate genes (Table 24.5).

Table 24.5 Mutation spectrum in genes of signaling and transcription factors responsible for microphthalmia

Gene	Variation	Phenotype	Novel/Reported	GenBank accession no./ dbSNP Id	References
GJA3	c.92T>A; p.Ile31Asn	Microphthalmia, microcornea, membranous cataract, and anterior capsular plaque	Novel	KX119953.1	[248]
GJA8	c.1018G>C; p.Glu340Gln	Bilateral anophthalmia	Novel	KU342018	[248]
SOX2	c.542C>A; .Pro181Gln	Isolated bilateral microphthalmia	Novel	KU342032	[248]
SOX2	c.541_542delinsGA; p.Pro181Glu	Bilateral microphthalmia, microcornea, learning disability, broad nasal bridge, and developmental delay	Novel	KU342033	[248]
BMP4	c.439+70_439+71insG	Isolated bilateral microphthalmia	Novel	KU342016	[248]

Table 24.5 (continued)

Gene	Variation	Phenotype	Novel/Reported	GenBank accession no./ dbSNP Id	References
FOXE3	c.691_693delGGG; p.231delGly	Bilateral microphthalmia, microcornea, congenital cataract	Novel	KU214460	[248]
RAX	c.786C>A; p.Gly262Gly	Bilateral anophthalmia	Novel	KU342027	[248]
BMP4	c.-7-39A>G	Bilateral anophthalmia	Reported	rs2761880	[249]
BMP4	c.455T>C; p.Val152Ala	Bilateral microphthalmia and microcornea	Reported	rs17563	[249]
FOXE3	c.-14G>A	Left eye microphthalmia, right eye anophthalmia	Reported	rs181190356	[250]
FOXE3	c.510C>T; p.Ala170Ala	Bilateral microphthalmia, microcornea, iris coloboma, chorioretinal coloboma	Reported	rs34082359	[250]
FOXE3	c.*72T>C	Bilateral microphthalmia, microcornea, iris coloboma	Reported	rs6666370	[250]
FOXE3	c.*77A>G	Bilateral microphthalmia, microcornea, iris coloboma	Reported	rs2820969	[250]
PAX6	c.781C>T; p.Arg261Ter	Glaucoma, microcornea, aniridia, sublux, conjunctival xerosis	Reported	Nil	[251, 252]
PITX3	c.285C>T; p.Ile95Ile	Bilateral microphthalmia, microcornea, iris coloboma, chorioretinal coloboma	Reported	rs2281983	[250]
RAX	c.132C>A; p.Asp44Glu	Bilateral microphthalmia, microcornea	Reported	rs2271733	[248]
RAX	c.882A>G; p.Gln294Gln	Left eye anophthalmia	Reported	rs7226481	[248]
SIX6	c.421C>A; p.His141Asn	Bilateral microphthalmia	Reported	rs33912345	[249]
VSX2	c.471C>T; p.Ser157Ser	Bilateral microphthalmia	Reported	rs35435463	[248]
VSX2	c.760+31_760+32insC	Bilateral microphthalmia	Reported	rs11383441	[248]

24.5.2.1 SOX2 Gene and Microphthalmia

The first novel SOX2 mutation c.542C>A; p.Pro181Gln was identified in a heterozygous condition in a 15-year-old female, her brother, mother, and grandmother with bilateral microphthalmia while the proband's father was normal. This mutation leads to the substitution of highly conserved amino acid proline at the amino acid position 181 to glutamine (p.Pro181Gln) in the SOX2 protein. The second novel SOX2 mutation (c.541_542delinsGA; p.Pro181Glu) was identified in a heterozygous condition in a 12-year-old sporadic case with bilateral microphthalmia, and microcornea with the broad nasal bridge, developmental delay, and learning disabilities. The family members of this patient were not available for screening. Both these SOX2 mutations were found to be deleterious and were absent in 100 controls. The position 181 of SOX2 protein was found to be highly conserved in different species using multiple sequence alignment. Using secondary structure prediction analysis, both these variations were found to cause the addition of a helix to the existing sheet resulting in a significant difference in coding position 181 of the secondary structure of SOX2.

It is important to note that two different mutations affecting the same amino acid position 181 of SOX2 protein could generate mild to severe phenotypes. This type of phenotypic variability in case of SOX2 mutations could be accredited to its considerable and widespread role in eye development as well as few specific features of the SOX2 protein. The SOX2 protein is composed of a highly conserved high mobility group (HMG) domain, which interacts with DNA, and a C-terminal transactivation domain, which works together with other proteins to activate the expression of its downstream target genes. SOX2 has been shown to have a low DNA-binding affinity and hence need partner factors (co-DNA-binding factors) to accomplish efficient and precise binding to the target DNA [245]. The mutation affecting the 181 amino acid position of SOX2 lies in the transactivation domain. Hence, it seems reasonable to believe that in order to accomplish its interaction with numerous other factors and to fulfill both the DNA-binding and transactivation functions, the phenotype produced by the SOX2 mutants may fluctuate to a great extent depending upon the type of SOX2 interactions throughout the different stages of eye development. Both these mutations were also found to modify the secondary structure of the SOX2 protein by the addition of a helix to the sheet. A minute change affecting this region might be fatal for the proper functioning of SOX2 and may produce unusual eye phenotypes as seen in both these patients harboring SOX2 mutation. This is so far the first report of a SOX2 mutation in microphthalmia patients from India. Also, we presume that amino acid position 181 of SOX2 protein could be a mutation hotspot for microphthalmia in the western Indian population and hence should be included in the screening panel of microphthalmia patients, especially in the Indian population.

24.5.2.2 Gap Junction Proteins: Novel Candidates of Microphthalmia

The lens transparency and homeostasis are maintained by the coupling of lens fiber cells to the intracellular gap junction channels formed by GJA3 and GJA8 [253, 254]. GJA3 and GJA8 are the most common cause of inherited cataracts in humans and mice. Gja3$^{-/-}$ knockout studies in mice suggest that GJA3 is crucial for maintaining lens transparency [255], while studies on Gja8$^{-/-}$ knockout mice have shown that it is essential for lens growth [256–259]. The Gja8$^{-/-}$ lenses show reduced epithelial proliferation and delayed maturation of fiber cells [257, 260]. Mutation in GJA8 gene has been associated with eye phenotypes such as microphthalmia, microcornea, and sclerocornea [261–263]. Prokudin et al. [264] identified a previously reported missense mutation (p.Arg198Gln) [265] in a patient with nuclear cataract and microcornea. Using next-generation sequencing, Ma et al. [266] identified de novo heterozygous variations (p.Gly94Glu and p.Gly94Arg) in two probands and one another variation, p.Asp51Asn, in another patient. These patients harbored eye phenotypes such as total sclerocornea and cataracts with or without microphthalmia.

GJA3 (Gap Junction Alpha 3) Gene Mutation and Microphthalmia

GJA3 maps to 13q12.11 and is a member of gap junction proteins. In our previous study, we reported two novel heterozygous variations, one each in GJA3 and GJA8 gene. The mutation, GJA3-p.Ile31Asn, was identified in a family of Indian descent having a bilateral congenital membranous cataract. The proband is a 14-year-old female having bilateral microphthalmia in addition to microcornea, and congenital membranous cataract with a thick anterior capsular plaque. This mutation was found to be deleterious by all the *in silico* analyses. The amino acid position 31 of GJA3 protein is highly conserved as revealed by multiple sequence alignment of GJA3 amino acids in different species. This variation was absent in 100 normal controls.

The GJA3-p.Ile31Asn mutation identified in this patient was also identified in the heterozygous condition in the patient's younger brother who had bilateral congenital cataract, microcornea, and anterior capsular plaque. Their father was blind due to trauma and did not harbor this mutation. Their mother could not be tested for the presence of this mutation as she is deceased. On interrogation and validation of reports, their mother was also found to have bilateral microphthalmia, microcornea, and congenital membranous cataract. No other members of this family had any abnormal ocular phenotypes. Given that their father was blind due to trauma during childhood, it is possible that that the proband and her brother might have inherited this mutation from their mother who also had the same eye phenotypes; however, it could not be tested as she is dead. The grandparents were normal and no other members in this family were found to harbor this phenotype. This signifies that the GJA3-p.Ile31Asp mutation is possibly a de novo mutation in the mother, which was inherited by both her children. It seems reasonable to believe that the GJA3-p.Ile31Asp mutation is the cause for autosomal dominant microphthalmia, congenital cataract, and microcornea observed in this family.

GJA3 plays an important role in coupling fiber cells in the central core of the lens [267]. Until now, more than 20 different mutations in the different domains of GJA3 have been reported to be linked with human autosomal inherited cataracts [268]. The majority of the GJA3 mutations that are linked with cataract have been identified in the transmembrane and the extracellular loop domains [267]. This mutation is present in the first transmembrane domain which is involved in the oligomerization into the connexon [261]. Therefore, it has the potential to influence the correct transport of proteins into the plasma membrane leading to abnormal development of the eye. Mutation in the first transmembrane domain of the GJA3 gene mutation has also been previously reported by four separate studies, out of which three reports are from India. This first transmembrane mutation was identified in nuclear pulverulent cataract (p.Phe32Leu), total cataract (p.Val28Met), posterior subcapsular cataract (p.Val28Val), and finely granular embryonal cataract (p.Arg33Leu) [262, 269–271]. This is the first study to show the association of GJA3 gene mutation with congenital membranous cataract, microphthalmia, and microcornea and therefore broadens the phenotype associated with the GJA3 mutation.

GJA8 (Gap Junction Alpha 8) Gene Mutation and Microphthalmia

GJA8 genes map to 1q21.1 and consist of two exons that encode 433 amino acid long protein. It is expressed in the mature lens and epithelial cells. It plays an essential role in eye development, especially in the differentiation of lens fiber cells, preservation of lens transparency, and normal growth of the eye [257, 272]. Further, it is responsible for the intracellular transport of ions and small molecular weight biomolecules in the lens. The structure of connexin consists of a cytoplasmic N-terminal domain, four transmembrane domains, two extracellular loops, a cytoplasmic loop between the transmembrane domain 2 and 3, and a C-terminal domain [273].

In our previous study, for the first time we reported a GJA8 missense variation in an anophthalmia patient (Table 24.5). The GJA8 mutation has been reported in different forms of congenital cataract, microphthalmia, microcornea, and sclerocornea [257, 266, 274–277].

24.6 Summary and Conclusion

Microphthalmia is a highly heterogeneous and incurable disease, owing to diverse mutations in the same or different genes. The majority of signaling and transcription factor genes show restricted spatiotemporal expression; however, a few of them reappear at different stages of eye development, controlling different developmental events. As these factors reappear at different events, tight genetic and epigenetic mechanisms might play a crucial role in normal eye development. Mutations in several genes especially transcription factors have been reported to cause congenital ocular anomalies. However, in the western Indian population such reports are scarce. Therefore, genetic studies of this kind are very important in the Indian subcontinent to identify the genetic mutations and their risk in disease manifestation in the next generation. Genetic data from such studies should help designing genetic diagnostics tool for the better clinical management of the individuals with varied severity of microphthalmia. Although GJA3 mutations have been shown to be associated with congenital cataract by various studies, this is the first report to link *GJA3* with congenital membranous cataract, microphthalmia, and microcornea. GJA3-p.Ile31Asn is the first mutation identified in patients with microphthalmia and cataract and suggests that GJA3 could be a novel candidate gene for microphthalmia. This broadens the mutation spectrum of GJA3 and highlights the importance of including GJA3 in the screening panel of microphthalmia, microcornea, and congenital cataract cases.

24.7 Future Perspectives

The future direction is aimed at identifying novel genes responsible for spectrum of microphthalmia phenotype by next-generation sequencing tools. Further, attempts could be made to understand the role of GJA3 mutations in microphthalmia conditions.

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25 Regional Differences in Prevalence of Myopia: Genetic or Environmental Effects?

Samantha S. Y. Lee and David A. Mackey

Abstract

It is well accepted that a myopia epidemic is occurring globally, with 50% of the global population predicted to have myopia by the year 2050. Parts of East and Southeast Asia, such as China, South Korea, and Singapore, have the most rapidly increasing rates of myopia compared to other parts of the world. The rate of rise in myopia differs between geographical locations and, even within a country, varying according to the level of urbanization of the studied region and between ethnic groups. Genetic factors are known to play a part in the myopia epidemic, with over 100 genes associated with myopia or its ocular traits already identified. Children in families where one or both parents have myopia are more likely to develop myopia. However, the epidemic is likely to be the result of environmental risk factors, such as higher levels of education, increased near work, and decreased time spent outdoors, rather than genetic factors. These environmental factors may predispose individuals who are genetically susceptible to develop myopia and may provide a target for early intervention and prevention.

Keywords

Myopia · Myopia epidemic · Myopia prevalence · Environmental risk factors · Genetics risk factors

S. S. Y. Lee (✉)
Centre for Ophthalmology and Visual Science (Incorporating the Lions Eye Institute), University of Western Australia, Nedlands, WA, Australia
e-mail: samanthalee@lei.org.au

D. A. Mackey
Centre for Ophthalmology and Visual Science (Incorporating the Lions Eye Institute), University of Western Australia, Nedlands, WA, Australia

Centre for Eye Research Australia, University of Melbourne, Royal Victorian Eye and Ear Hospital, East Melbourne, VIC, Australia

School of Medicine, Menzies Research Institute Tasmania, University of Tasmania, Hobart, TAS, Australia
e-mail: d.mackey@utas.edu.au

25.1 Introduction

Over the past few decades, there has been a rapid increase in the prevalence of myopia globally, with the epidemic most marked and well defined in East Asia [1–8]. While the reported increase in myopia prevalence may be partly attributed to improvements in research methodology (e.g., defining ametropia using refraction values rather than visual acuity) [9] and better access to healthcare (which increases diagnostic rates of

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myopia), recent findings from large-scale meta-analyses [10, 11] provide clear evidence of a true myopia epidemic.

Myopia has long been known to run in families, suggesting a genetic predisposition. Individuals are more likely to be having myopia if either or both of their parents have myopia [12–15]. However, genetic traits are usually passed down from one generation to another, thus, heredity is unlikely to explain the current epidemic. We must therefore refocus our attention on environmental or lifestyle factors—what has changed that might have led to the dramatic rise in myopia prevalence? What is the extent of the role of genetics in an individual's susceptibility to the environmental effects of myopia? This chapter shall explore these issues as well as discuss the factors underlying the variations in myopia prevalence across the Asia-Pacific.

25.2 Prevalence

In the year 2010, the global prevalence of myopia—defined as spherical equivalent ≤ -0.50 diopter (D)—was estimated to be 1950 million (28.3% of the world population) [11]. The most striking numbers come from the industrialized countries of Asia, including Korea [1–3], Singapore [16–19], China [4–6], and Taiwan [7, 8], where up to 80–97% of young adults have myopia. In fact, although less than one-quarter of the world's population reside in these four countries, they account for almost 40% of the global myopia burden [11]. Other parts of Southeast and East Asia have the next highest rates of myopia, with prevalence rates in adults of approximately 50% [20, 21] and 20% [22, 23], respectively. In Australia, prevalence of myopia has been reported to be 15–23% in adults [24–26].

At the extreme end of the spectrum, the Oceanic regions have the lowest myopia rates in the world, where less than 5% of the population has myopia [11, 27, 28]. Clearly, myopia rates vary considerably within the Asia-Pacific region. Two important factors contribute to this variation: differences in the environment (specifically urbanization) and genetic predisposition, which can be revealed by examining differences between ethnic groups.

25.2.1 Variation with Urbanization

It is well established that urbanization has a strong association with myopia [10, 29–33]. Large countries are inherently more likely to have conspicuous disparities in the level of urbanization and provide an opportunity to tease out its effect. For example, in Xiamen, China, 19.3% of children who attend schools in the city had myopia, almost triple the prevalence of the more rural parts of the region (6.6%) [33]. A similar pattern was noted in Yangxi, where 50% of adolescents living in the urbanized areas had myopia compared to 33% living in the rural areas [34]. This trend is not limited to children. As noted in the Beijing Eye Study [32], older adults (40–90 years old) living in the urban district of Haidian tend to be more myopic than those living in the village area of Yufa, with mean spherical equivalents of −0.55 D and −0.06 D, respectively. Other population-based studies [4, 5, 35–37] in urban China have consistently reported higher myopia prevalence than those in rural regions [6, 38, 39].

Likewise, the prevalence of myopia is higher in urban districts of South Korea [2, 3], Taiwan [8], and Vietnam [40] than in more rural areas. In Seoul, the highly metropolitan capital of South Korea, 96.5% of 19-year-old males have myopia [3]. In Jeju, a rural region of South Korea, the reported myopia prevalence in 19-year-old males is slightly less alarming but nevertheless high at 83.3% [2]. Similarly, in Taiwan, 15-year-old children in large cities (e.g., Taipei, Kaohsiung) had higher rates of myopia compared to the "remote," "hilly," and "aboriginal" areas of the country (78.2–84.5% vs. 31.8–82.5%) [8].

In South Asia, on the other hand, the link between urbanization and myopia prevalence is more ambiguous. For example, in the rural Mahabubnagar district of Andhra Pradesh, India, myopia prevalence in school children was

reported to be 4.1% [41], which is not dissimilar to that found in some urban areas of India (e.g., Hyderabad: 4.4%, Srinagar: 4.7%). (An exception may be Delhi, India's capital and largest urban area by population, where myopia rates in school children of 7.4–13.1% have been reported.) Other population-based studies in India [42, 43], as well as in Bangladesh [22] and Pakistan [44], also did not find myopia prevalence significantly different between urban and rural populations. On the other side of the Asia-Pacific, the Melbourne Visual Impairment Project [26] (Victoria, Australia) similarly did not find a significant difference in myopia rates between rural and urban residents of the state.

However, many of these studies [22, 26, 42–45] in older populations excluded participants who had undergone cataract surgery, which usually aims for an emmetropic refraction endpoint. This may have underestimated true myopia prevalence, particularly in urban areas where the residents are more likely to have better access to and be able to afford healthcare services. This is in contrast to rural areas, where cataract treatments are less accessible [46–48]. The relatively higher incidence of nuclear cataracts, which are associated with myopic shifts, may have resulted in (perhaps artificially) increased myopia prevalence [42, 43]. Indeed, in the Andhra Pradesh eye disease study [43], while myopia prevalence was found to be higher in those residing in rural regions, when the data were reanalyzed after excluding those with nuclear cataracts grade ≥ 2 (according to the Lens Opacities Classification System), the odds ratio for myopia was increased to 2.5 in urban compared to rural areas.

The body of literature demonstrates a clear variation in myopia prevalence according to the level of urbanization, even within country borders, in the Asia-Pacific. There still remains a lack of updated reports on myopia rates in some parts of the Asia-Pacific, such as in Japan and New Zealand where the degree of urbanization varies widely within borders. In addition, because of the increasing popularity of cataract and refractive surgery, studies on myopia prevalence in older populations need to consider the presence of cataracts and history of cataract or refractive surgery in their analyses. Population-based studies should address these in order to determine whether the demand for myopia control and eye care delivery is keeping up with the population demand.

25.2.2 Variation with Ethnicity

It is well recognized that myopia is much more prevalent in Asia than in other parts of the world [11, 30, 49, 50]. Children of Asian descent living in Western countries [49, 50], including Australia which is part of the Asia-Pacific [12, 24], have also consistently had higher rates of myopia compared to those of Caucasian descent. Most of these studies compared Asians with Caucasians; however, myopia prevalence can also differ between specific Asia ethnic groups. For example, as discussed above, East Asia has a higher rate of myopia than South Asia. Given the geographical and cultural difference between the two regions (e.g., Chinese individuals tend to have a stronger emphasis on academic attainment), it may be hard to determine whether the difference is a result of ethnic background (i.e., more of a genetic factor) or environment. This is where studies arising from multiethnic cities, such as Singapore and Malaysia, may be particularly valuable for isolating specific contributing factors.

Within both Singapore and Malaysia, there are three major ethnic groups, all of Asian origin: Chinese (which may represent East Asians), Indians (South Asians), and Malays (Southeast Asians). These different ethnicities have been resident for generations; mass immigration from India and China occurred prior to World War II, while Malays are native to these regions [51]. We can assume that individuals from different ethnic groups in these countries are exposed to similar environments, and therefore tease out differences in myopia prevalence between ethnic groups that may be less dependent on geographical factors.

Indeed, studies based in Singapore [19, 52–54] and Malaysia [52] have consistently found that the Chinese have the highest rates of myopia and the Malays the lowest rates (Table 25.1). A cross-sectional study in Malaysia [55] also reported that mean refractive error in Chinese individuals with myopia increases rapidly from approximately −2.0 D to 4.0 D between 10 and 20 years of age, while the severity of myopia in Malaysians tends to remain relatively constant at −2.5 D across all age groups. However, the cross-sectional nature of the latter study cannot provide solid data on myopia progression with increasing age.

Cultural differences, rather than genetics, could still account for these differences in myopia prevalence. For example, the Chinese culture traditionally has a greater emphasis on academic attainment, while the Malays tend to be overrepresented in blue-collar occupations [56]. Indeed, a population-based study [53] of young male conscripts in Singapore reported that their Chinese counterparts had higher levels of education than the Malays, which may partly explain the higher myopia rates in the former group (odds ratios [OR] for myopia and high myopia of 1.3 and 3.0, respectively). However, even after adjusting for education levels, the Chinese sample still remained significantly more likely to be myopic than the Malays (ORs for myopia and high myopia 1.1 and 1.5, respectively). In the same study [53], the Indian sample was also more likely to have high myopia than the Malays after adjusting for education (OR 1.2). This finding showed that, even when different groups of individuals are raised in relatively homogenous environments, both genetic factors and education play a key role in myopia development.

Table 25.1 Prevalence of myopia according to ethnicity in multiethnic countries

Study (author, date)	Sample	Prevalence of myopia and high myopia (%)		
		Chinese	Indians	Malay
Au Eong et al., 1993[a] (Singapore)	• *N* = 110,236. • Young male conscripts • 15–25 years old	• Myopia: 48.5% • High myopia: 26.8%	• Myopia: 30.4% • High myopia: 14.1%	• Myopia: 24.5% • High myopia: 9.5%
Wu et al., 2001[b] (Singapore)	• *N* = 15,095 • Young male conscripts • 16–25 years old	• Myopia: 82.2% • High myopia: 14.8%	• Myopia: 68.7% • High myopia: 6.3%	• Myopia: 65.0% • High myopia: 5.0%
Saw et al., 2006[c, d] (Singapore and Malaysia)	• *N* = 3714. • School children • 7–9 years old	Singapore • Myopia: 40.1% • High myopia: - Malaysia • Myopia: 30.9% • High myopia: -	Singapore • Myopia: 34.1% • High myopia: - Malaysia • Myopia: 12.5% • High myopia: -	Singapore • Myopia: 22.1% • High myopia: - Malaysia • Myopia: 9.2% • High myopia: -
Koh et al., 2014 (Singapore)	Young male conscripts 1996–1997 cohort: • *N* = 15,085 • 16–25 years old 2009–2010 cohort: • N = 28,908. • 17–29 years old	1996–1997 cohort: • Myopia: 82.2% • High myopia: 14.8% 2009–2010 cohort: • Myopia: 85.9% High myopia: 18.2%	1996–1997 cohort: • Myopia: 68.6% • High myopia: 6.3% 2009–2010 cohort: • Myopia: 74.6% High myopia: 7.3%	1996–1997 cohort: • Myopia: 64.9% • High myopia: 13.1% 2009–2010 cohort: • Myopia: 70.7% High myopia: 14.7%

[a]Myopia and high myopia defined as ametropia with unaided visual acuities of 6/18 and 6/60, respectively, as it was assumed that myopia prevalence is closely related to ametropia in that sample
[b]Myopia and high myopia defined as spherical equivalents of < −0.5 D and < −6.0 D, respectively
[c]Myopia defined as spherical equivalent of ≤ −0.5 D
[d]Ethnicity-specific prevalence of high myopia was not reported

25.3 Genetic Factors

As mentioned in an earlier section, the prevalence of myopia varies substantially between ethnicities, strongly suggesting a role for heredity in myopia. Indeed, twin and family studies of myopia and its related biometry (e.g., axial length and corneal curvature) have shown these traits to be highly heritable [57]. However, estimates of the heritability of myopia vary widely between studies, ranging from 12% to over 90% [58–64], although these differences may partly be attributed to environmental differences between samples.

Before the advent of genome-wide association studies (GWAS) in the late 2000s, linkage and candidate gene studies for myopia had already identified several loci and genes related to myopia [65], although many of these findings have not been replicated in more contemporaneous experiments. Nonetheless, these studies, which were conducted in families and high-risk groups, have identified the MYP 1 to 20 loci for high myopia [66, 67] and up to 50 other genes and independent loci [68–76].

Just within the past decade, GWAS has further identified more than 100 new loci and genes associated with myopia. The first GWAS for myopia was conducted by Nakanishi et al. [77], who identified that the *BLID* and *LOC399959* genes in chromosome 11q24 were associated with pathological myopia. Several other GWAS similarly focused on high or pathological myopia, which is known to be strongly influenced by genetics [78], and in East Asian populations. To date, such studies have identified or confirmed high myopia genes located at chromosomes 4q25 [79–82], 4q22−q27 [83], and 13q12 [84], among others.

Close collaborations between scientists globally have led to larger GWAS meta-analyses, including those by the Consortium for Refractive Error And Myopia (CREAM) [85] and 23andMe [86]. By 2015, these two groups had already identified over 40 novel genes and replicated several others (Table 25.2). Incredibly, although the two groups used different methods of defining myopia and analysis, many of the genes identified or replicated by one were also found by the other [87]. Furthermore, most of the novel genes identified by either CREAM or 23andME were later validated by subsequent studies [88–93]. As the decade comes to a close, combined efforts by the CREAM and 23andMe have brought the total number of refractive error genes to 161 and counting (Table 25.2).

25.3.1 Ocular Endophenotypes of Myopia

In 1994, Zadnik et al. [94] demonstrated that myopia could result from ocular characteristics transmitted from parent to child, i.e., be genetically inherited. The researchers observed that children (6–14 years old) with two parents with myopia tended to have longer axial lengths, even before the onset of myopia, compared to those with only one or no parent with myopia. A cross-sectional study by Ip et al. [12] later reported similar findings. However, findings from a one-year longitudinal study [95] in East Asian children suggested that parental myopia influences the rate of axial elongation rather than the axial length per se. Regardless, these observations are a few of many examples of the heredity of the ocular traits in myopia and the possibility that multiple genes underlie these traits.

Indeed, several loci for endophenotypes of myopia have also been identified in GWAS. The first studies involved individuals of East and Southeast Asian heritage [96, 97] and found a link between axial length and the *ZC3H11B* gene. This finding was later confirmed by Cheng et al. [91], who identified an additional eight novel genes associated with axial length (*ALPPL2, C3orf26, CD55, GJD2, LAMA2, MIP, RSPO1, ZNRF3*). Five of these genes (*ALPPL2, CD55, GJD2, LAMA2, ZC3H11B*) have also been linked with refractive errors in previous studies [85, 86]. This latter finding suggests that many of the refractive error genetic variants previously identified do not affect refractive error directly, but rather, they control the ocular endophenotypes that influence refractive error. Subsequently,

Table 25.2 Myopia single-nucleotide polymorphisms (and gene[s]) identified or replicated by CREAM and 23andMe [85, 86], presented in

11:128787963:D (*KCNJ5*)
11:30281122:D (*FSHB, ARL14EP*)
17:56619441:I (*C17orf47*)
22:23069851:I (*DKFZp667J0810, abParts*)
4:80993942:CTT_C (*ANTXR2*)
8:121622778:D (*SNTB1*)
rs10003846 (*C4orf22, BMP3*)
rs10104039 (*BIN3, EGR3*)
rs10122788 (*MVB12B*)
rs10187371 (*ZEB2*)
rs10458138 (*LOC100508120*)
rs10500355 (*RBFOX1*)
rs10511652 (*SH3GL2, ADAMTSL1*)
rs1064583 (*COL10A1*)
rs10760673 (*TGFBR1*)
rs10853531 (*SLC14A2*)
rs10880855 (*ARID2*)
rs10887262 (*RGR*)
rs11088317 (*NRIP1, USP25*)
rs11101263 (*FRMPD2*)
rs11118367 (*LYPLAL1*)
rs11145465 (*TJP2*)
rs11160044 (*NDUFB1*)
rs11178469 (*PTPRR*)
rs11202736 (*RNLS*)
rs11210537 (*HIVEP3*)
rs1150687 (*ZNF192P1, TRNA_Ser*)
rs11589487 (*AK097193, BC030753*)
rs11602008 (*LRRC4C*)
rs116226959 (*SCAND3*)
rs11654644 (*B4GALNT2, TRNA_Gln*)
rs11723482 (*PCAT4, ANTXR2*)
rs117735470 (*ST8SIA1, C2CD5*)
rs11802995 (*KIRREL*)
rs11952819 (*ZNF366*)
rs1207782 (*LINC00340*)
rs12193446 (*BC035400, LAMA2*)
rs1237670 (*HP08777*)
rs12405776 (*PLD5*)
rs12451582 (*NOG, C17orf67*)
rs12526735 (*KCNQ5*)
rs12667032 (*DPP6*)
rs12883788 (*AKAP6, NPAS3*)
rs12898755 (*APH1B*)
rs12965607 (*MYO5B*)
rs13069734 (*ZBTB38*)
rs1358684 (*SEMA3D, GRM3*)
rs1359543 (*RCBTB1*)
rs144370238 (*SSR1, CAGE1*)
rs1454776 (*GALNT15*)
rs1532278 (*CLU*)
rs1550094 (*PRSS56*)
rs1555075 (*RALY*)
rs1556867 (*5S_rRNA, PBX1*)
rs1649068 (*BICC1*)
rs17032696 (*CAMKMT, SIX3*)
rs17125093 (*TTC8, TRNA_Ala*)
rs17382981 (*CYP26A1, MYOF*)
rs17400325 (*PDE11A*)
rs17428076 (*HAT1, METAP1D*)
rs1790165 (*NTM*)
rs1858001 (*C4BPA, CD55*)
rs1928175 (*LINC00340*)
rs1954761 (*GRIA4*)
rs1969091 (*TMC3, MEX3B*)
rs1983554 (*MEI1, bK250D10.C22.8*)
rs1994840 (*C4orf22*)
rs2116093 (*BC043573*)
rs2143964 (*BMP4, CDKN3*)
rs2150458 (*PCBP3, COL6A1*)
rs2155413 (*DLG2*)
rs2166181 (*RASGEF1B, U6*)
rs2225986 (*LINC00862*)
rs2229742 (*NRIP1*)
rs2276560 (*EIF4E2, EFHD1*)
rs2303635 (*AMOTL2*)
rs2326823 (*BC035400*)
rs235770 (*BMP2*)
rs2573081 (*PDE11A*)
rs2573210 (*PRSS56*)
rs2573232 (*ALPPL2, ALPI*)
rs2745953 (*CD34*)
rs2753462 (*JB175233, C14orf39*)
rs2823097 (*NRIP1, USP25*)
rs28471081 (*RBFOX1*)
rs284816 (*ST18, FAM150A*)
rs2855530 (*BMP4*)
rs28658452 (*MYCN, SNORA40*)
rs28891973 (*NCOA2, TRAM1*)
rs2908972 (*SHISA6*)
rs297593 (*GPD2*)
rs3110134 (*SNORA51, CA8*)
rs3138137 (*BLOC1S1-RDH5, RDH5*)
rs34539187 (*FBN1*)
rs35337422 (*RD3L*)
rs36024104 (*LRFN5*)
rs41393947 (*PNPT1, EFEMP1*)
rs4237284 (*C10orf11*)
rs4260345 (*THRB*)
rs4687586 (*CACNA1D*)
rs4764038 (*GRIN2B*)
rs478304 (*RNASEH2C, AP5B1*)
rs4793501 (*KCNJ2, BC039327*)
rs4795364 (*MED1*)
rs4805962 (*KCTD15, LSM14A*)
rs4808962 (*GATAD2A*)
rs4894529 (*FNDC3B*)
rs511217 (*METTL15, KCNA4*)
rs524952 (*GOLGA8B, GJD2*)
rs5442 (*GNB3*)
rs56055503 (*MAF, DYNLRB2*)
rs56075542 (*BC040861, PABPC1P2*)
rs60884546 (*VIPR2*)
rs62070229 (*MYO1D, TMEM98*)
rs629631 (*PRTN3*)
rs6420484 (*TSPAN10*)
rs6433704 (*PDE11A*)
rs6495367 (*RASGRF1*)
rs6753137 (*FAM150B, TMEM18*)
rs7042950 (*RORB*)
rs7107014 (*HNRNPKP3, API5*)
rs7122817 (*DSCAML1*)
rs7207217 (*BC039327, D43770*)
rs72621438 (*SNORA51, CA8*)
rs72655575 (*SNORA51, CA8*)
rs7275394 (*TIAM1*)
rs72826094 (*TCF7L2*)
rs7337610 (*FLT1*)
rs73730144 (*VIPR2*)
rs7449443 (*FLJ16171, DRD1*)
rs745480 (*LRIT2, LRIT1*)
rs74764079 (*BMP3*)
rs7624084 (*ZBTB38*)
rs7662551 (*LOC100506035, PCAT4*)
rs7692381 (*C4orf22, BMP3*)
rs7737179 (*TMEM161B-AS1, LINC00461*)
rs7744813 (*KCNQ5*)
rs7747 (*ANTXR2*)
rs7789096 (*VIPR2, NONE*)
rs7829127 (*ZMAT4*)
rs78627037 (*abParts*)
rs7895108 (*KCNMA1*)
rs79266634 (*RBFOX1*)
rs7941828 (*MPPED2*)
rs7968679 (*PZP*)
rs7971334 (*PDE3A*)
rs80253120 (*CDRT15*)
rs807037 (*KAZALD1*)
rs8075280 (*POLR2A, TNFSF12*)
rs837323 (*PCCA*)
rs9295499 (*CDKAL1*)
rs931302 (*NONE, SETMAR*)
rs9388766 (*L3MBTL3*)
rs9395623 (*TFAP2D, TFAP2B*)
rs9416017 (*DNAJB12*)
rs9516194 (*GPC5, GPC6*)
rs9517964 (*ZIC2, PCCA*)
rs9547035 (*LINC00333, LINC00351*)
rs9606967 (*AK123891, SYN3*)
rs9680365 (*GRIK1*)
rs9681162 (*AK124857, LMCD1*-AS1)

[a]Those in bold were found by both the CREAM and 23andMe

other GWAS identified several other genes for axial length and corneal curvature [98, 99], which are known key associates of refractive error.

25.3.2 Inheritance Pattern

Although the numerous genes identified by GWAS for myopia and other traits of refractive error suggest a polygenic inheritance, earlier family studies suggest many genes, including the MYP 1–20 genes [66, 67], inherit an autosomal-dominant (AD) pattern [100–103]. An exception is the X-linked heterozygous gene *ARR3* mutations in females [104]. All patterns of inheritance have been identified for ocular and systemic syndromes associated with high myopia. Mutations in genes causing cone-rod dystrophies may have inheritance patterns of AD (e.g., *CRX, GUCA1A, GUCY2D*), autosomal recessive (AR; e.g., *ABCA4, CNGA3*), and X-linked recessive (XLR; e.g., *CACNA1F, PRGR*) [105]. Likewise, congenital stationary night blindness may be caused by mutant genes with inheritance patterns of AD (e.g., *RHO, GNAT1*), AR (e.g., SAG, RHOK), and XLR (*CACNA1F*) [106]. Other ocular syndromes similarly have multiple associated mutated genes that are transmitted in varying patterns. Ocular and systemic genetic syndromes are outside the scope of this chapter; see Pinazo-Duran et al. [107] for a brief review on ocular and systemic associations of eye conditions.

25.4 Environmental Factors

In the late 1960s, myopia prevalence in a group of Inuit in Alaska suddenly increased; no myopia was present in their parents and earlier generations [109]. This epidemic, which arose within a single generation within families, occurred after these families moved from more isolated parts of the state to the relatively urbanized city of Barrow, Alaska, where schools have been established for decades. This was one of the earliest indications that myopia can be driven by environmental change. Since then, numerous other studies have found a strong association between environmental factors and myopia.

Two major environmental factors have been widely accepted as the main culprits of myopia: increasing education and reduced time spent outdoors, and these two factors are often strongly linked. That is, children who spend more time indoors doing near work tend to do so at the expense of outdoor activities. Therefore, these risk factors may not be independent of each other. Indeed, in a Sydney-based study [110], higher near work/outdoor activity hour ratio (more time spent on near work relative to being outdoors) was a stronger risk factor for myopia than just near work per se. However, as demonstrated in numerous studies [111–116], time spent outdoors is not simply an inverse value of time spent on near work and studying. For example, Rose et al. [111] reported that even though school children in Sydney spent more time on near work than those in Singapore, the former also spent more time outdoors and have a lower prevalence of myopia than the latter. Therefore, near work (or education) and time spent outdoors are each likely to be an independent risk factor for myopia. These risk factors are discussed in more detail below.

25.4.1 Education

Education, whether measured in terms of duration (years of formal education) [54, 117], academic scores [15, 118], or highest level achieved [18, 34, 117], has consistently been found to have a positive link with myopia. The jurisdictions in the Asia-Pacific with the highest academic performance (Shanghai-China, Hong Kong-China, South Korea, Japan, Singapore, and Taiwan) are also the areas with the highest myopia prevalence in the world [119]. Even in parts of the Asia-Pacific with lower myopia prevalence, such as India [120] and Australia [25, 26], higher education levels are strongly associated with higher rates of myopia.

However, the direction of causality in the relationship between education and myopia is unknown. Is myopia a result of spending more

time engaging in academic activities or do individuals with myopia have a tendency to spend more time on near work and studying because of their reduced distance vision? Recently, using Mendelian randomization [121], researchers [122] were able to work out that every additional year spent in formal education would result in a −0.27 D increase in myopia ($p < 0.001$). On the other hand, myopia was not found to have an effect on education ($p = 0.60$). This was the first study [122] to provide concrete evidence that more education is a causal factor for myopia. Given that this study [122] was based in Great Britain, which has a mainly Caucasian population, future studies should examine this causal relationship in other ethnic groups and populations, including those in the Asia-Pacific.

25.4.2 Near Work and Hyperopic Defocus

Although we are now fairly certain that increased education is a risk factor of myopia (and not the reverse), the mechanism underlying this relationship remains unclear. Our best theory for this link is that with increased near work associated with education, the demand for long durations of near focusing may result in hyperopic defocus at the central [123] and/or peripheral retina [124–126], stimulating axial growth. Findings from animal experiments support this hyperopic defocus theory [127–129].

Using this defocus concept, in theory, myopic defocus should have the opposite effect or at least slow down axial growth and minimize myopia progression. Indeed, animal studies [128, 129] have shown that myopic defocus retards axial elongation. Conversely in humans, producing a myopic defocus in children's eyes by undercorrecting their myopia has proven to be counterproductive, speeding up myopia progression instead [130–132]. Yet, if the hyperopic defocus theory is erroneous, why do optical methods of myopia control (e.g., progressive spectacles, peripheral defocus modifying lenses, undercorrection) that are based on this theory show some evidence of efficacy (albeit with small effect sizes) in slowing myopia progression? For now, hyperopic defocus during near work remains our best working theory for the link between education and myopia. Nonetheless, more research is needed to fill our gaps in our understanding of this link between near work and myopia.

25.4.3 Time Spent Outdoors

The evidence for the inverse relationship between time spent outdoors and myopia, which only emerged fairly recently, is extensive and undeniable [24, 111–113, 133–140]. In a meta-analysis of 7 cross-sectional studies, Sherwin et al. [140] estimated that every additional hour of time spent outdoors per week would result in a 2% decrease in odds of developing myopia. Studies from the Asia-Pacific [13, 112, 135, 141, 142] generally reported that children without myopia tend to spend approximately 2–3 more hours per week outdoors than those with myopia. There is an exception for very young Asian children (less than 6 years old), where the difference in time spent outdoors did not significantly differ between those with or without myopia, suggesting that earlier-onset myopia has more of a genetic basis rather than environmental [13, 143].

Other cross-sectional [13, 111, 112, 137, 142] and longitudinal studies [113, 134, 141] also confirmed that more time spent outdoors (self-reported) was associated with less myopia. Objective measurements of sun exposure, such as the amount of light exposure measured with actigraphy devices [135], area of conjunctival autofluorescence [144], and serum vitamin D levels [24, 145–148], have all also demonstrated that outdoor exposure has a strong inverse association with myopia. Moreover, studies in the USA [149, 150] and China [151] found that myopic refractive error and axial length tends to increase more rapidly during the winter months, when day length is shortest, than during summer. One

Japanese study [152] similarly reported that axial growth is slowest during summer, although refractive error progression did not vary with seasonal change.

The inverse association between myopia and time spent outdoors prompts a few vital questions. First, to gain the protective effect against myopia, is it necessary to engage in physical activities and sports (which are generally performed outdoors) or is it sufficient to just spend time outdoors? A number of studies [134, 153–155] reported that myopia is associated with less participation in sports and physical activities. The Singapore Cohort Study of Risk Factors for Myopia (SCORM) demonstrated that indoor sports activities were not associated with myopia whereas those performed outdoors were, strongly suggesting that physical activity itself is not protective against myopia. Findings from a prospective study in Brisbane [135] confirmed that individuals with myopia tend to spend less time outdoors than those with emmetropia, as measured with actigraphy watches, but levels of physical activities were similar between the two groups of individuals.

Second, is this relationship between outdoor activity and myopia driven by a tendency for those with myopia to stay indoors more as a result of their poor distance vision or is outdoor activity actually protective against myopia? Recent studies [156–158] in East Asia found that intervention programs that encourage children to spend more time outdoors significantly reduce myopia incidence, progression, and axial growth (Table 25.3). A meta-analysis [159] estimated that this increase in time spent outdoors would result in a reduced myopic progression be −0.30 D over the course of 3 years. From these inter-

Table 25.3 Clinical trials using more time spent outdoors as an intervention

Study	Region	Sample	Intervention(s)	Trial duration	Findings
He et al., 2015	Guangzhou, China	$N = 1903$; 6–7 years old	40-min additional class of outdoor activity; parents encouraged to engage children in outdoor activities outside school	3 years	Intervention group vs control group: • Myopia incidence: 30.4% vs 39.5% (group difference $p < 0.01$). • Refractive error shift: −1.42 D vs. −1.59 D ($p = 0.04$). • Axial growth: 0.95 mm vs 0.98 mm ($p = 0.07$).
Jin et al., 2015	Sujiatun, China	$N = 3051$; 6–14 years old	20-min additional recess time outside classroom	1 year	Intervention group vs control group: • Myopia incidence: 3.7% vs 8.5% ($p = 0.05$). • Refractive error shift: −0.10 D vs. −0.27 D ($p < 0.01$). • Axial growth: 0.16 mm vs 0.21 mm ($p < 0.01$).
Wu et al., 2013	Taiwan	$N = 471$; 7–11 years old	Encouraged to have recess outside classroom	1 year	Intervention group vs control group: • Myopia incidence: 8.4% vs 17.7% ($p < 0.01$). • Refractive error shift: −0.25 D vs. 0.38 D ($p = 0.03$).
Yi a Li 2011	Changsha, China	$N = 80$; 7–11 years old	Limit near- and middle-vision activities to less than 30 h/week, and engage in outdoors activities to more than 14–15 h/week	2 years	Intervention group vs control group: • Refractive error shift: −0.38 D vs. 0.52 D ($p < 0.01$).

vention studies, we may infer a protective effect of outdoor time against myopia rather than those with myopia having an aversion to being outdoors.

25.5 Gene–Environment Interaction

In addition to environmental or genetic factors as independent associates of myopia, it is crucial to understand the gene–environment interaction effects on refractive error. Are some individuals susceptible to developing myopia even in the absence of environmental risk factors? Do others harbor genes "protective" against myopia?

Similar to how the evidence of environmental effects on myopia emerged only relatively recently, it was not until the mid-2010s that interest in studying gene–environment interactions for myopia became more widespread. In a study of Amish families, Wojciechowski et al. [65] found that some SNPs are associated with refractive error in those with lower education but not in those who have attained a higher level of education (bachelor's degree or higher). This suggests that some individuals are indeed more susceptible to developing refractive error despite not being exposed to known major environmental factors. A meta-analysis [160] of five cohort studies in Singapore found that the myopia genes *DNAH9, GJD2,* and *ZMAT4* were more strongly associated with myopia and axial length in those who had attained higher secondary education or higher compared to those with lower levels of education. Several other studies have also revealed interactions between genetic load and education [161] or near work [161, 162].

25.6 Summary

Myopia prevalence is increasing worldwide but varies substantially between and even within regions. East Asia is experiencing the largest burden of myopia, with a prevalence of 78–97% in young adults living in metropolitan areas [28]. Myopia is highly heritable—individuals whose parents have myopia are at increased risk of developing myopia. Indeed, GWAS and other genetic studies have identified over 100 genes associated with myopia or its ocular traits. However, these genes combined can only explain 7.8% of the phenotypic variance of refractive error [108], suggesting that more genes are yet to be identified and/or environmental factors may be key drivers of the myopia epidemic.

Environmental factors of myopia include increased education, increased amount of near work, and reduced time spent outdoors. These characteristics are more often exhibited by urban dwellers, which may explain the higher myopia prevalence in metropolitan versus rural areas. While the evidence on reduced time outdoors as a risk factor for myopia emerged only fairly recently, it is extensive and undeniable. Several clinical trials in China and Taiwan have even demonstrated that increasing time outdoors could be a viable treatment method for myopia control.

As studies on the genetic and environmental factors of myopia progress, our understanding of myopiagenesis will grow. Genetic studies can potentially facilitate identification of at-risk individuals, while environmental studies can provide information on modifiable myopia risk factors to minimize vision loss from myopia complications.

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26

Consortium for Refractive Error and Myopia (CREAM): Vision, Mission, and Accomplishments

Annechien E. G. Haarman, Milly S. Tedja, CREAM Consortium, Magda A. Meester-Smoor, Jaakko Kaprio, David A. Mackey, Jeremy A. Guggenheim, Christopher J. Hammond, Caroline C. W. Klaver, and Virginie J. M. Verhoeven

Details of authors from "CREAM Consortium" are included in Appendix.

CREAM Consortium

A. E. G. Haarman · M. S. Tedja
M. A. Meester-Smoor
Department of Ophthalmology, Erasmus Medical Center, Rotterdam, The Netherlands

Department of Epidemiology, Erasmus Medical Center, Rotterdam, The Netherlands
e-mail: a.haarman@erasmusmc.nl; m.tedja@erasmusmc.nl; m.meester-smoor@erasmusmc.nl

J. Kaprio
Institute for Molecular Medicine, University of Helsinki, Helsinki, Finland

Department of Public Health, University of Helsinki, Helsinki, Finland
e-mail: jaakko.kaprio@helsinki.fi

D. A. Mackey
Centre for Eye Research Australia, Ophthalmology, Department of Surgery, University of Melbourne, Royal Victorian Eye and Ear Hospital, Melbourne, Victoria, Australia

Department of Ophthalmology, Menzies Institute of Medical Research, University of Tasmania, Hobart, Tasmania, Australia

Centre for Ophthalmology and Visual Science, Lions Eye Institute, University of Western Australia, Perth, Western Australia, Australia
e-mail: d.mackey@utas.edu.au

J. A. Guggenheim
School of Optometry and Vision Sciences, Cardiff University, Cardiff, UK
e-mail: GuggenheimJ1@cardiff.ac.uk

C. J. Hammond
Section of Academic Ophthalmology, School of Life Course Sciences, King's College London, London, UK
e-mail: chris.hammond@kcl.ac.uk

C. C. W. Klaver
Department of Ophthalmology, Erasmus Medical Center, Rotterdam, The Netherlands

Department of Epidemiology, Erasmus Medical Center, Rotterdam, The Netherlands

Department of Ophthalmology, Radboud University Medical Center, Nijmegen, The Netherlands

Institute of molecular and clinical ophthalmology, Basel, Switzerland
e-mail: c.c.w.klaver@erasmusmc.nl

V. J. M. Verhoeven (✉)
Department of Ophthalmology, Erasmus Medical Center, Rotterdam, The Netherlands

Department of Epidemiology, Erasmus Medical Center, Rotterdam, The Netherlands

Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, The Netherlands
e-mail: v.verhoeven@erasmusmc.nl

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Abstract

The Consortium for Refractive Error and Myopia (CREAM) is an international collaboration founded to increase knowledge on the genetic background of refractive error and myopia. The consortium was established in 2011 and consists of >50 studies from all over the world with epidemiological and genetic data on myopia endophenotypes. Due to these efforts, almost 200 genetic loci for refractive error and myopia have been identified. These genetic risk variants mostly carry low risk but are highly prevalent in the general population. The genetic loci are expressed in all retinal cell layers and play a role in different processes, e.g., in phototransduction or extracellular matrix remodeling. The work of CREAM over the years has implicated the major pathways in conferring susceptibility to myopia and supports the notion that myopia is caused by a light-dependent retina-to-sclera signaling cascade. The current genetic findings offer a world of new molecules involved in myopiagenesis. However, as the currently identified genetic loci explain only a fraction of the high heritability, further genetic advances are needed. It is recommended to expand large-scale, in-depth genetic studies using complementary big data analytics, to consider gene-environment effects by thorough measurements of environmental exposures, and to focus on subgroups with extreme phenotypes and high familial occurrence. Functional characterization of associated variants is simultaneously needed to bridge the knowledge gap between sequence variance and consequence for eye growth. The CREAM consortium will endeavor to play a pivotal role in these future developments.

Keywords

Myopia · Refractive error · Genetics · GWAS GxE interactions

Acronyms

ALSPAC	Avon Longitudinal Study of Parents and Children
AREDS	Age-Related Eye Disease Study
BMES	Blue Mountain Eye Study
CA repeats	Cytosine–Adenine repeats
CREAM	Consortium for Refractive Error and Myopia
GEWIS	genome-environment-wide interaction studies
GCTA	genome-wide complex trait analysis
GWAS	genome-wide association studies
GxE	gene–environment interaction
KORA	Cooperative Health Research in the Region Augsburg
MR	Mendelian randomization
nmol/l	nanomole/liter
OMIM	Online Mendelian Inheritance in Man database
SNP	single-nucleotide polymorphism
SSGAC	Social Science Genetic Association Consortium
WES	whole-exome sequencing
WGS	whole-genome sequencing

26.1 Key Points

1. Refractive errors including myopia are caused by a complex interplay between many common genetic factors and environmental factors (near work, outdoor exposure).
2. Early linkage studies and candidate gene studies have identified up to 50 loci and genes, but findings remained mostly unverified in replication studies.
3. Large consortia, e.g., CREAM, performing genome-wide association studies (GWAS) enabled the identification of common genetic variants associated with refractive error and myopia.
4. The CREAM consortium and 23andMe published findings from GWAS separately and later combined studies in a GWAS meta-analysis. Together they identified 161 common variants for refractive error, explaining ~8% of the phenotypic variance of this trait. As the majority of the phenotypic variance of refractive errors is still unexplained, larger sample sizes are required with deeper coverage of the genome.

5. Polygenic risk scores based on these variants indicate that persons at high genetic risk have an up to 40× increased risk of myopia compared to persons at low genetic risk.
6. The genetic loci appear to play a role in synaptic transmission, cell–cell adhesion, calcium ion binding, cation channel activity, and the plasma membrane. Many are involved in light-dependent processes, which was confirmed by pathway analysis, and related to cell cycle and growth pathways.
7. Genome-environment-wide interaction studies (GEWIS) assessing variant × education interaction effects identified 9 other loci. Evidence for statistical interaction was also found; those at profound genetic risk with higher education appeared particularly more susceptible to myopia.
8. The ultimate aim of genetic studies is to discern the molecular signaling cascade and open up new avenues for intervention.

26.2 Introduction

Common myopia is caused by a complex interplay between environmental and genetic factors [1]. This most prevalent form of myopia, in contrast to, e.g., syndromic myopia, is presumably caused by many genes each with a very small effect. Large sample sizes were needed to identify these genes which required a collaboration between genetic researchers in ophthalmology worldwide. Therefore, the Consortium for Refractive Error and Myopia (CREAM), consisting of >50 refractive error studies providing both genetic and phenotypical information of large populations with both European and Asian genetic background, was founded in 2011. The CREAM consortium enabled conducting large genome-wide association studies (GWAS) which led to the identification of almost 200 genetic factors for myopia and refractive error (Fig. 26.1).

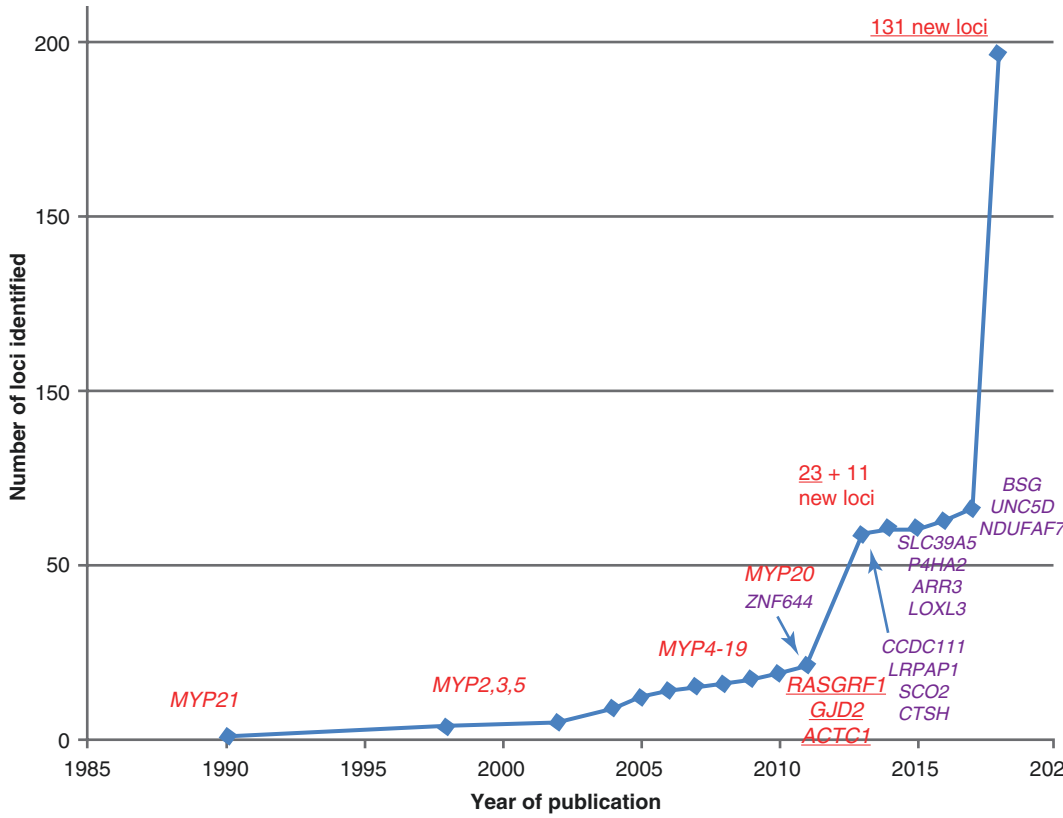

Fig. 26.1 Historic overview of myopia gene finding. Overview of myopia gene finding in a historic perspective. Genes identified using whole-exome sequencing are marked as purple. Other loci (linkage studies, GWAS) are marked as red. Loci identified in the CREAM consortium are underlined

To understand the process which has led to the foundation of CREAM, we first explain in detail several approaches of gene discovery from linkage analysis to next-generation sequencing which have been conducted in the last decades. This overview is based on a recent review [2]. Myopia is a highly heritable trait, although estimates vary from 15 to 98% [3–8]. The search for genes that underlie the heritability of myopia was initiated by linkage studies among families and high-risk groups. These studies have highlighted the heterogeneous genetic etiology of refractive error. Searches for additional candidate genes yielded positive associations with several genes. Although these studies yielded associations, a general lack of validation emerged across studies. A more powerful and successful approach to identify genetic variants for common myopia is GWAS, which robustly investigates numerous single-nucleotide polymorphisms across the genome in large populations [9–17]. This approach requires large sample sizes and joint forces of studies, which eventually led to the foundation of CREAM.

Despite the efforts of CREAM, the genetic variants identified to date only explain about 8% of the heritability of myopia [18]. It will be challenging to unravel this hidden heritability and find new genes which ultimately will lead to the understanding of the complete pathophysiologic mechanism of myopia. At the end of this chapter, suggestions for future research approaches are proposed [2, 18].

26.3 Heritability

Eighty years ago, Sir Duke-Elder was one of the first to recognize a "hereditary tendency to myopia" [25]. Since then, evidence for familial aggregation has been delivered by various familial clustering, twin, and offspring studies, [9, 15–17] and a genetic predisposition became more widely recognized. Strikingly, the estimates of myopia heritability vary widely among studies, with values as low as 10% [4, 26] found in a parent–offspring study in Eskimos, to as high as 98% in a study of female twin pairs [5, 6, 8] (Table 26.1). Differences in study design and method of analysis may account for this, but it is also conceivable that the phenotypic variance determined by heritable factors is high in settings where environmental triggers are limited, and low where they are abundant.

Table 26.1 Correlation in myopia between different pairs

	Study	Correlation coefficient (95% CI)
	Dirani et al. [19]	0.61
Monozygous twin pair	Dirani et al. [19]	0.61
	Hammond et al. [20]	0.86–0.83
	Lyhne et al. [6]	0.91 (0.85–0.95)
Dizygous twin pair	Dirani et al. [19]	0.16
	Hammond et al. [20]	0.47–0.48
	Lyhne et al. [6]	0.44 (0.21–0.63)
Sibling pair	Guggenheim et al. [21]	0.447 (0.314–0.564)
	Peet et al. [22]	0.35 (0.24–0.46) (refractive error)
	Klein et al. [23]	0.328
Parent–offspring pair	Lim et al. [24]	0.296 (0.266–0.326) (refractive error)
Second-degree relatives	Klein et al. [23]	0.123

Twin studies also estimated a high heritability for most of the individual biometric parameters [23, 27]. Correlations between corneal curvature and axial length were at least 64%, [28] suggesting a considerable genetic overlap between the parameters.

Studies addressing the inheritance structure of myopia and its endophenotypes identified several models, mostly a combination of additive genetic and environmental effects [7, 20, 27, 29]. Genome-wide complex trait analysis (GCTA) using high-density genome-wide single-nucleotide polymorphism (SNP) genotype information was performed in young children from the Avon Longitudinal Study of Parents and Children (ALSPAC) study, and results suggested that common SNPs explained approximately

35% of the variation in refractive error between unrelated subjects [30]. SNP heritability calculated by LD score regression in the CREAM consortium was 21% in Europeans but only 5% in Asians, which could be due to the low representation of this ancestry [18].

In conclusion, the genetic component of myopia and ocular biometry is well recognized but its magnitude varies in studies depending on the population being studied, the study design, and methodology. It is important to note that the recent global rise of myopia prevalence is unlikely to be due to genetic factors, but the degree of myopia may still be under genetic control [31].

26.4 Linkage Studies

A number of linkage studies for myopia was performed in families and high-risk groups before the GWAS era (Fig. 26.1) [32]. Linkage studies have searched for cosegregation of genetic markers (such as cytosine-adenine (CA) repeats) with the trait through pedigrees and has been successfully applied for many Mendelian disorders [33]. In families with an autosomal-dominant inheritance pattern of myopia, this approach helped to identify several independent loci for (high) myopia: MYP 1-20 [32, 34–36] as well as several other loci [37–42]. Fine mapping of several of these loci led to candidate genes, such as the *IGF1* gene located in the MYP3 locus [1]. Although validation of the same markers failed in these candidate genes, other variants appeared associated with common myopia, suggesting genetic overlap between Mendelian and complex myopia [43]. Linkage studies using a complex inheritance design found five additional loci [44–48].

With the development of new approaches for gene finding, linkage analysis with microsatellite-markers became unfashionable. Nevertheless, segregation analysis of a variant or region in pedigrees is still a common procedure for fine-mapping or dissection of disease haplotypes.

26.5 Secondary Syndromic Myopia

Myopia can accompany other systemic or ocular abnormalities. The secondary syndromic myopias are generally monogenic and have a wide spectrum of clinical presentations. Table 26.2 summarizes the known syndromic and ocular

Table 26.2 Overview of secondary syndromic forms of myopia

A. Systemic syndromes associated with myopia	
Title	Gene and inheritance pattern
Acromelic frontonasal dysostosis	*ZSWIM6 (AD)*
Alagille syndrome	*JAG1 (AD)*
Alport syndrome	*COL4A5 (XLD); COL4A3 (AR/AD)*
Angelman syndrome	*UBE3A (IP); CH*
Bardet–Biedl syndrome	*ARL6; BBS1; BBS2; BBS4; BBS5; BBS7; BBS9; BBS10; BBS12; CEP290; LZTFL1; MKKS; MKS1; SDCCAG8; TMEM67; TRIM32; TTC8; WDPCP (AR)*
Beals syndrome	*FBN2 (AD)*
Beaulieu–Boycott–Innes syndrome	*THOC6 (AR)*
Bohring–Opitz syndrome	*ASXL1 (AD)*
Bone fragility and contractures; arterial rupture and deafness	*PLOD3 (AR)*
Branchiooculofacial syndrome	*TFAP2A (AD)*
Cardiofaciocutaneous syndrome	*MAP2K2 (AD)*
Cohen syndrome	*VPS13B (AR)*
Cornelia de Lange syndrome	*NIPBL (AD); HDAC8 (XLD)*
Cowden syndrome	*PTEN (AD)*
Cranioectodermal dysplasia	*IFT122 (AR)*

(continued)

Table 26.2 (continued)

A. Systemic syndromes associated with myopia	
Title	Gene and inheritance pattern
Cutis laxa	*ATP6V0A2; ALDH18A1 (AR)*
Danon disease	*LAMP2 (XLD)*
Deafness and myopia	*SLITRK6 (AR)*
Desanto–Shinawi syndrome	*WAC (AD)*
Desbuquois dysplasia	*CANT1 (AR)*
Donnai–Barrow syndrome	*LRP2 (AR)*
DOORS	*TBC1D24 (AR)*
Ehlers–Danlos syndrome	*COL5A1 (AD); PLOD1 (AR); CHST14 (AR); ADAMTS2 (AR); B3GALT6 (AR); FKBP14 (AR)*
Emanuel syndrome	CH
Fibrochondrogenesis	*COL11A1 (AR)*
Gyrate atrophy of choroid and retina with/without ornithinemia	*OAT (AR)*
Hamamy syndrome	*IRX5 (AR)*
Homocystinuria	*CBS (AR)*
Joint laxity; short stature; myopia	*GZF1 (AR)*
Kaufman oculocerebrofacial syndrome	*UBE3B (AR)*
Kenny–Caffey syndrome	*FAM111A (AD)*
Kniest dysplasia	*COL2A1 (AD)*
Knobloch syndrome	*COL18A1 (AR)*
Lamb–Shaffer syndrome	*SOX5 (AD)*
Lethal congenital contracture syndrome	*ERBB3 (AR)*
Leukodystrophy	*POLR1C; POLR3A; POLR3B; GJC2 (AR)*
Linear skin defects with multiple congenital anomalies	*NDUFB11; COX7B (XLD)*
Loeys–Dietz syndrome	*TGFBR1; TGFBR2 (AD)*
Macrocephaly/megalencephaly syndrome	*TBC1D7 (AR)*
Marfan syndrome	*FBN1 (AD)*
Marshall syndrome	*COL11A1 (AD)*
Microcephaly with/without chorioretinopathy; lymphedema; and/or mental retardation	*KIF11 (AD)*
Mohr–Tranebjaerg syndrome	*TIMM8A (XLR)*
Mucolipidosis	*GNPTAG (AR)*
Muscular dystrophy	*TRAPPC11; POMT; POMT1; POMT2; POMGNT1; B3GALNT2; FKRP; DAG1; FKTN(AR)*
Nephrotic syndrome	*LAMB2 (AR)*
Noonan syndrome	*A2ML1; BRAF; CBL; HRAS; KRAS; MAP2K1; MAP2K2; NRAS; PTPN11; RAF1; RIT1; SOS1; SHOC2; SPRED1 (AD)*
Oculocutaneous albinism	*TYR (AR)*
Oculodentodigital dysplasia	*GJA1 (AR)*
Pallister–Killian syndrome	CH
Papillorenal syndrome	*PAX2 (AD)*
Peters-plus syndrome	*B3GLCT (AR)*
Pitt–Hopkins syndrome	*TCF4 (AD)*
Pontocerebellar hypoplasia	*CHMP1A (AR)*
Poretti–Boltshauser syndrome	*LAMA1 (AR)*
Prader–Willi syndrome	NDN (PC); SNRPN (IP); CH
Pseudoxanthoma elasticum	*ABCC6 (AR)*
Renal hypomagnesemia	*CLDN16; CLDN19 (AR)*
SADDAN	*FGFR3 (AD)*
Schaaf–Yang syndrome	*MAGEL2 (AD)*

Table 26.2 (continued)

A. Systemic syndromes associated with myopia	
Title	Gene and inheritance pattern
Schimke immunoosseous dysplasia	*SMARCAL1 (AR)*
Schuurs–Hoeijmakers syndrome	*PACS1 (AD)*
Schwartz–Jampel syndrome	*HSPG2 (AR)*
Sengers syndrome	*AGK (AR)*
Short stature; hearing loss; retinitis pigmentosa and distinctive facies	*EXOSC2 (AR)*
Short stature; optic nerve atrophy; and Pelger–Huet anomaly	*NBAS (AR)*
SHORT syndrome	*PIK3R1 (AD)*
Short-rib thoracic dysplasia with/without polydactyly	*WDR19 (AR)*
Shprintzen–Goldberg syndrome	*SKI (AD)*
Singleton–Merten syndrome	*IFIH1 (AD)*
Small vessel brain disease with/without ocular anomalies	*COL4A1 (AD*
Smith–Magenis syndrome	*RAI1 (AD)*
Spastic paraplegia	*HACE1 (AR)*
Split hand/foot malformation	CH
Stickler syndrome	*COL2A1 (AD); COL11A1 (AD); COL9A1 (AR); COL9A2 (AR)*
Syndromic mental retardation	*SETD5 (AD); MBD5 (AD); USP9X (XLD); NONO (XLR); RPL10 (XLR); SMS (XLR); ELOVL4 (AR); KDM5C (XLR)*
Syndromic microphthalmia	*OTX2; BMP4 (AD)*
Temtamy syndrome	*C12orf57 (AR)*
White–Sutton syndrome	*POGZ (AD)*
Zimmermann–Laband syndrome	*KCNH1 (AD)*

B. Ocular syndromes associated with myopia	
Title	Gene and inheritance pattern
Achromatopsia	*CNGB3 (AR)*
Aland Island eye disease	*GPR143 (XLR)*
Anterior segment dysgenesis	*PITX3 (AD)*
Bietti crystalline corneoretinal dystrophy	*CYP4V2 (AD)*
Blue cone monochromacy	*OPN1LW; OPN1MW (XLR)*
Brittle cornea syndrome	*ZNF469; PRDM5 (AR)*
Cataract	*BFSP2; CRYBA2; EPHA2 (AD)*
Colobomatous macrophthalmia with microcornea	CH
Cone dystrophy	*KCNV2 (AD)*
Cone–rod dystrophy	*C8orf37 (AR); RAB28 (AR); RPGR (XLR); CACNA1F (XLR)*
Congenital microcoria	CH
Congenital stationary night blindness	*NYX (XLR); CACNA1F (XLR); GRM6 (AR); SLC24A1 (AR); LRIT3 (AR); GNB3 (AR); GPR179 (AR)*
Ectopia lentis et pupillae	*ADAMTSL4 (AR)*
High myopia with cataract and vitreoretinal degeneration	*P3H2 (AR)*
Keratoconus	*VSX1 (AD)*
Leber congenital amaurosis	*TULP1 (AR)*
Microcornea, myopic chorioretinal atrophy, and telecanthus	*ADAMTS18 (AR)*
Microspherophakia and/or megalocornea, with ectopia lentis and/or secondary glaucoma	*LTBP2 (AR)*
Ocular albinism	*OCA2 (AR)*
Primary open-angle glaucoma	*MYOC; OPTN (AD)*
Retinal cone dystrophy	*KCNV2 (AR)*
Retinal dystrophy	*C21orf2 (AR); TUB (AR)*

(continued)

Table 26.2 (continued)

B. Ocular syndromes associated with myopia	
Title	Gene and inheritance pattern
Retinitis pigmentosa	*RP1 (AD); RP2 (XLR); RPGR (XLR); TTC8 (AR)*
Sveinsson chorioretinal atrophy	*TEAD1 (AD)*
Vitreoretinopathy	*ZNF408 (AD)*
Wagner vitreoretinopathy	*VCAN (AD)*
Weill–Marchesani syndrome	*ADAMTS10 (AR); FBN1 (AD); LTBP2 (AR); ADAMTS17 (AR)*

AD autosomal dominant, *AR* autosomal recessive, *XLR* X linked recessive, *XLD* X linked dominant, *CH* chromosomal, *IP* imprinting defect

conditions that present with myopia [49]. Among these disorders are many mental retardation syndromes, such as Angelman (Online Mendelian Inheritance in Man database (OMIM) #105830), Bardet–Biedl (OMIM #209900), Cohen (OMIM #216550), and Pitt–Hopkins syndrome (OMIM #610954). Myopia can also be a characteristic feature in heritable connective tissue disorders, such as Marfan (OMIM #154700), Stickler (OMIM #108300, #604841, #614134, #614284), Weill–Marchesani syndrome (OMIM #277600, #608328, #614819, #613195), and several types of Ehlers–Danlos syndrome (OMIM #225400, #601776).

A number of inherited retinal dystrophies also present with myopia, most strikingly X-linked retinitis pigmentosa caused by mutations in the *RPGR*-gene (retinal G protein-coupled receptor) (see reference [42, 46] for common gene acronyms) and congenital stationary night blindness [47]. Other eye disorders accompanied by myopia are ocular albinism (OMIM #300500) and Wagner vitreoretinopathy (OMIM #143200).

The majority of the genes causing syndromic forms of myopia have not (yet) been implicated in common forms of myopia, except for *COL2A1 (collagen type II alpha 1 chain)* [48, 49] and *FBN1* (*fibrilin 1*) [18, 50]. However, a recent study screened polymorphisms located in and around genes known to cause rare syndromic myopia and found them to be overrepresented in GWAS studies on refractive error and myopia [51]. This implies that while rare, pathogenic mutations in these genes have a profound impact on the eye, more benign polymorphisms may only have subtle effects on ocular biometry and refractive error.

26.6 Candidate Gene Studies

Candidate genes are generally selected based on their known biological, physiological, or functional relevance to the disease. Although sometimes highly effective, this approach is limited by its reliance on existing knowledge. Another caveat not specific for this approach is that genetic variability across populations can make it difficult to distinguish normal variation from disease-associated variation [12]. Additionally, candidate gene studies are very prone to publication bias and therefore published results are highly selected.

Numerous genes have been investigated in candidate gene studies for refractive error traits. Table 26.3 summarizes all studies that reported statistically significant associations for myopia or ocular refraction. Genes that encode collagens (*COL1A1* and *COL2A*1 [48, 49]), transforming growth factors (*TGFβ1, TGFβ2*, and *TGIF1 (TGFβ induced factor homeobox 1)* [52–54]), hepatocyte growth factor and its receptor (*HGF* and *CMET* [55–57]), insulin-like growth factor (*IGF1* [58, 59]), matrix metalloproteinases (*MMP1, MMP2, MMP3, MMP9, MMP10* [60, 61]), the lumican gene (*LUM* [62]), and the ocular developmental gene *PAX6* [63] all showed promise in candidate gene studies. Unfortunately, like myopia linkage studies, these studies generally lacked validation by independent studies. Meta-analyses combining data from several candidate gene studies provided evidence for a consistent association between a single SNP in the *PAX6* gene and extreme and high myopia [64]. Meta-analyses of the *LUM and IGF1* genes did not confirm an association [65, 66].

Table 26.3 Summary of candidate gene studies reporting positive association results with myopia

Gene	Study	Ethnicity	Independent confirmation	Replication in GWAS
APLP2	Tkatchenko et al. 2015 [138]	Caucasian	–	–
BMP2K	Liu et al. 2009 [163]	Chinese	–	–
CHRM1	Lin et al. 2009 [164]	Han Chinese	X [165]	–
CHRM1	Guggenheim et al. 2010 [166]	Caucasian	X [165]	–
CMET	Khor et al. 2009 [57]	Chinese	–	–
COL1A1	Inamori et al. 2007 [167]	Japanese	–	–
COL2A1	Mutti et al. 2007 [48]	Caucasian	–	–
COL2A1	Metlapally et al. 2009 [49]	Caucasian	–	–
CRYBA4	Ho et al. 2012 [168]	Chinese	–	–
HGF	Han et al. 2006 [56]	Han Chinese	–	–
HGF	Yanovitch et al. 2009 [169]	Caucasian	–	–
HGF	Veerappan et al. 2010 [55]	Caucasian	–	–
IGF1	Metlapally et al. 2010 [59]	Caucasian	–	–
LUM	Wang et al. 2006 [62]	Chinese	–	–
LUM	Chen et al. 2009 [170]	Han Chinese	–	–
LUM	Lin et al. 2010 [170, 171]	Chinese	–	–
LUM	Guggenheim et al. 2010 [166]	Caucasian	–	–
MFN1	Andrew et al. 2008 [172]	Caucasian	X [173]	–
MMP1	Wojciechowski et al. 2010 [137]	Amish	–	–
MMP1	Wojciechowski et al. 2013 [61]	Caucasian	–	–
MMP10	Wojciechowski et al. 2013 [61]	Caucasian	–	–
MMP2	Wojciechowski et al. 2010 [137]	Amish	–	–
MMP2	Wojciechowski et al. 2013 [61]	Caucasian	–	–
MMP3	Hall et al. 2009 [60]	Caucasian	–	–
MMP9	Hall et al. 2009 [60]	Caucasian	–	–
MYOC	Tang et al. 2007 [60, 174]	Chinese	–	–
MYOC	Vatavuk et al. 2009 [175]	Caucasian	–	–
MYOC	Zayats et al. 2009 [176]	Caucasian	–	–
PAX6	Tsai et al. 2008 [177]	Chinese	–	–
PAX6	Ng et al. 2009 [178]	Han Chinese	–	–
PAX6	Han et al. 2009 [179]	Han Chinese	–	–
PAX6	Miyake et al. 2012 [180]	Japanese	–	–
PAX6	Kanemaki et al. 2015 [181]	Japanese	–	–
PSARL	Andrew et al. 2008 [172]	Caucasian	–	–
SOX2T	Andrew et al. 2008 [172]	Caucasian	–	
TGFβ1	Lin et al. 2006 [52]	Chinese	–	X [18]
TGFβ1	Zha et al. 2009 [182]	Chinese	–	X [18]
TGFβ1	Khor et al. 2010 [58]	Chinese	–	X [18]
TGFβ1	Rasool et al. 2013 [183]	Indian	–	X [18]
TGFβ2	Lin et al. 2009 [53]	Han Chinese	–	–
TGIF	Lam et al. 2003 [54]	Chinese	–	–
TGIF1	Ahmed et al. 2014 [54, 184]	Indian	–	–
LAMA1	Zhao et al. 2011 [185]	Chinese	–	–
UMODL1	Nishizaki et al. 2009 [186]	Japanese	–	–

26.7 Genome-Wide Association Studies (GWAS)

Since the first genome-wide association study (GWAS) in 2005 [67], over 3000 human GWAS have examined over 1800 diseases and traits, and thousands of SNP associations have been found. This has greatly augmented our knowledge of human genetics and complex diseases [13]. GWAS genotyping arrays can identify millions of SNPs across the genome in one assay; these variants are generally common and mostly not protein coding. Effect sizes of SNPs associated

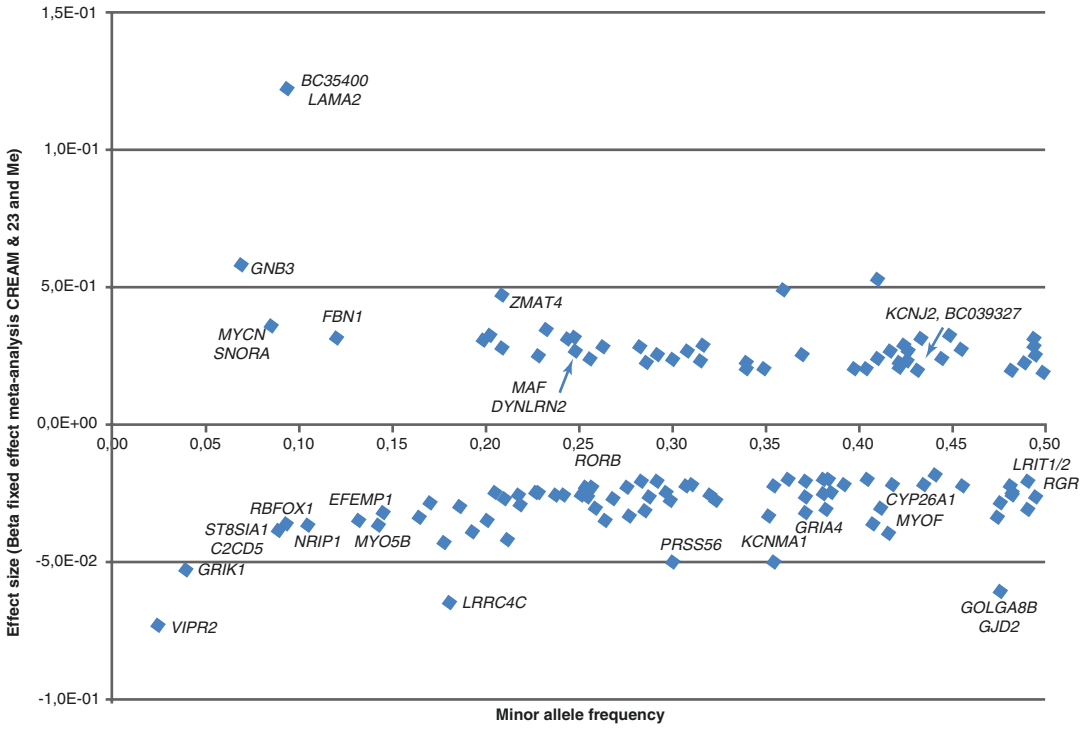

Fig. 26.2 Effect sizes of common and rare variants for myopia and refractive error. Overview of SNPs and annotated genes found in the most recent GWAS meta-analysis (Tedja et al. 2018). *X*-axis displays the minor allele frequency of each SNP; *Y*-axis displays the effect size of the individual SNP, calculated using the Z-score of the fixed effect, equally weighted meta-analysis

with disease are mostly small, requiring very large study samples to reach statistical significance [12, 13]. Fortunately, technological advances have lowered the costs of genotyping considerably over the years, [68] and GWAS on hundreds of thousands of individuals are becoming more common.

26.7.1 GWAS of Refractive Errors and Myopia

GWAS for myopia have been performed using myopia as a dichotomous outcome or refractive error as a quantitative trait. Several endophenotypes have also been considered: spherical equivalent, axial length, corneal curvature, and age of diagnosis of myopia.

Figure 26.2 provides an overview of all associated loci and nearby genes, their frequency and effect sizes.

26.7.1.1 Myopia Case–Control Design

The case–control design using (high) myopia as a dichotomous outcome has been especially popular in East Asia. The first GWAS case–control study was performed in a Japanese cohort in 2009 [69]. It comprised 830 cases of pathologic myopia (defined as axial length > 26 mm) and 1911 controls from the general population. The strongest association was located at 11q24.1, approximately 44 kb upstream of the *BLID (BH3-like motif containing, cell death inducer)* gene, and conferred odds of higher myopia of 1.37 (95% confidence interval (CI) 1.21–1.54). Subsequently, a GWAS meta-analysis of two eth-

nic Chinese cohorts was performed in 287 cases of high myopia (defined as ≤ −6D) and 673 controls. The strongest association was for an intronic SNP within the *CTNND2 (catenin delta 2)* gene on 5p15.2 [70]. Neither of these associations met the conventional GWAS threshold ($p \leq 5 \times 10^{-8}$) for statistical significance due to small sample size. Nevertheless, the locus at 5p15 encompassing the *CTNND2* gene was later confirmed by other Asian studies [71–73].

Li et al. studied 102 high myopia cases (defined as ≤ −8D with retinopathy) and 335 controls in an ethnic Chinese population [74]. The strongest association ($p = 7.70 \times 10^{-13}$) was a high-frequency variant located in a gene desert within the MYP11 myopia linkage locus on 4q25. In a similar ethnic Han Chinese population of 419 high myopia cases (≤ −6D) and 669 controls, Shi et al. identified the strongest association ($p = 1.91 \times 10^{-16}$) at an intronic, high-frequency variant within the *MIPEP (mitochondrial intermediate peptidase)* gene on 13q12 [74, 75]. Neither hit has been replicated, even in studies with similar design, phenotypic definition, and ethnic background.

In 2013, two papers reported loci for high myopia in Asian populations, and these were successfully replicated. Shi et al. studied a Han Chinese population of 665 cases with high myopia (≤ −6D) and 960 controls [76]. Following two-stage replication in three independent cohorts, the most significantly associated variant ($p = 8.95 \times 10^{-14}$) was identified in the *VIPR2 (vasoactive intestinal peptide receptor 2)* gene within the MYP4 locus, followed by three other variants within a linkage disequilibrium block in the *SNTB1 (syntrophin beta 1)* gene ($p = 1.13 \times 10^{-8}$ to 2.13×10^{-11}). Khor et al. reported a meta-analysis of four GWAS including 1603 cases of "severe" myopia and 3427 controls of East Asian ethnicity [77]. After replication and meta-analysis, the *SNTB1* gene was confirmed, and a novel variant within the *ZFHX1B* gene (also known as *ZEB2 (zinc finger E-box binding homeobox 2)*) reached genome-wide significance ($p = 5.79 \times 10^{-10}$).

In 2018, a pathologic myopia case–control study was performed in cohorts of Asian ancestry, using participants with −5.00 D or more myopia with an axial length > 26 mm. Fundus photographs were graded pathologic or non-pathologic ($N_{cases} = 828$, $N_{controls} = 3624$). The researchers found a novel genetic variant in the *CCDC102B (coiled-coil domain containing 102B)* locus ($p = 1.46 \times 10^{-10}$), which was subsequently replicated in an independent cohort ($p = 2.40 \times 10^{-6}$). This gene is strongly expressed in the retinal pigment epithelium and choroid. As myopic maculopathy is the primary cause of blindness in high myopia, further functional investigation could be valuable [78].

In Europe, a French case–control GWAS was performed on 192 high myopia cases (≤−6D) and 1064 controls, and a suggestive association was identified within the MYP10 linkage locus, 3 kb downstream of *PPP1R3B (protein phosphatase 1 regulatory subunit 3B)*. However, this association did not reach genome-wide statistical significance, and no previously reported loci were replicated [79]. Later, in 2016, the direct-to-consumer genetic testing company 23andMe (Mountain View, CA, USA) published a large GWAS on self-reported myopia ($N_{cases} = 106{,}086$ and $N_{controls} = 85{,}757$; all European ancestry), and identified more than 100 novel loci for myopia [80]. Since this study was intended for association analyses between traits, precise locus definitions, post-GWAS quality control, and replication were not performed.

26.7.1.2 Quantitative Design on Spherical Equivalent

Studies that considered refractive error as a quantitative trait, and included subjects from the general population who displayed the entire range of refractive error, have been more successful. In 2010, the first GWAS for spherical equivalent were carried out in two European populations: a British cohort of 4270 individuals and a Dutch cohort of 5328 individuals [81, 82]. Two loci surpassed the GWAS threshold and were replicated: one near the *RASGFR1* gene on 15q25.1 ($p = 2.70 \times 10^{-09}$) and the other near *GJD2* on 15q14 ($p = 2.21 \times 10^{-14}$). Subsequently, a meta-analysis was performed on 7280 individuals with refractive error from five different cohorts, which

included various ethnic populations across different continents, and findings were replicated in 26,953 samples. A novel locus including the *RBFOX1* gene on chromosome 16 reached genome-wide significance ($p = 3.9 \times 10^{-9}$) [83].

These collaborations paved the way for the formation of a large consortium to achieve higher statistical power for gene finding. The Consortium for Refractive Error and Myopia (CREAM) was established in 2010 and included researchers and cohorts from the US, Europe, Asia, and Australia. Its first collaborative work was the replication of SNPs in the previously identified 15q14 loci [84]. Other studies followed this approach and confirmed 15q14 as well as the 15q25 locus [85, 86]. Subsequently, CREAM conducted a GWAS meta-analysis based on HapMapII imputation [87] with 35 participating studies comprising 37,382 individuals of European descent and 12,332 of Southeast Asian ancestry with data on GWAS and spherical equivalent. This study enabled replication of *GJD2*, *RASGRF1,* and RFBOX1 and identification of 23 novel loci at genome-wide significance*: BICC1, BMP2, CACNA1D, CD55, CHD7, CHRNG, CYP26A1, GRIA4, KCNJ2, KCNQ5, LOC100506035, LAMA2, MYO1D, PCCA, TJP2, PTPRR, SHISA6, PRSS56, RDH5, RORB, SIX6, TOX,* and *ZMAT472* [88].

Meanwhile, 23andMe performed a contemporaneous large GWAS on 55,177 individuals of European descent by using a survival analysis, based on the first release of 1000G [89] (a catalog of human genetic variation). Its analysis was based on self-reported presence of myopia and age of spectacle wear as a proxy for severity. 23andMe also replicated *GJD2*, *RASGRF1*, *and RFBOX1* and identified 11 new loci: *BMP3, BMP4, DLG2, DLX1, KCNMA1, LRRC4C, PABPCP2, PDE11A, RGR, ZBTB38, ZIC2* [90]. Of the 22 loci discovered by CREAM, 8 were replicated by 23andMe, while 16 of the 20 loci identified by 23andMe were confirmed by CREAM. This was surprising as the studies used very different phenotyping methods. Additionally, the effect sizes of 25 loci were very similar, despite analyses on different scales: dioptres for CREAM and hazard ratios for 23andMe [91]. After these two publications, replication studies provided validation for *KCNQ5, GJD2, RASGRF1, BICC1, CD55, CYP26A1, LRRC4C, LAMA2, PRSS56, RFBOX1, TOX, ZIC2, ZMAT4*, and *B4GALNT2* in per-SNP analyses, and for *GRIA4, BMP2, BMP4, SFRP1, SH3GL2,* and *EHBP1L1* in gene-based analyses [92–97].

Although CREAM and 23andMe found a large number of loci, only ~3% of the phenotypic variance of refractive error was explained [88, 90]. Larger GWAS meta-analyses were clearly needed, and the two large studies combined efforts. This new GWAS meta-analysis was based on the phase 1 version 3 release of 1000G, included 160,420 participants, and findings were replicated in the UK Biobank (95,505 participants). Using this approach, the number of validated refractive error loci increased to 161. A high genetic correlation between Europeans and Asians (>0.78) was found, implying that the genetic architecture of refractive error is quite similar for Europeans and Asians. Taken together, these genetic variants accounted for 7.8% of the explained phenotypic variance, leaving room for improvement. Even so, polygenic risk scores, which are constructed by the sum of effect sizes of all risk variants per individual depending on their genotypes, were well able to distinguish individuals with hyperopia from those with myopia at the lower and higher deciles. Interestingly, those in the highest risk decile had a 40-fold-greater risk of myopia. The predictive value of these risk scores for myopia vs. hyperopia, adjusted for age and sex, was an AUC = 0.77 (95% CI = 0.75–0.79).

The next step will include GWAS on even larger sample sizes. Although this will improve the explained phenotypic variance, it is unlikely that GWAS will uncover the entire missing heritability. SNP arrays do not include rare variants, nor do they address gene–environment and gene–gene interactions, or epigenetic effects [98].

26.7.1.3 GWAS on Refractive Error Endophenotypes

As myopia is mostly due to increased axial length, researchers have used this parameter as a myopia proxy or "endophenotype." The first

axial length GWAS examined 4944 individuals of East and Southeast Asian ancestry, and a locus on 1q41 containing the zinc-finger pseudogene *ZC3H11B* reached genome-wide significance ($p = 4.38 \times 10^{-10}$) [83, 99]. A much larger GWAS meta-analysis of axial length comprised 12,531 Europeans and 8216 Asians [94]. This study identified eight novel genome-wide significant loci (*RSPO1, C3orf26, LAMA2, GJD2, ZNRF3, CD55, MIP, ALPPL2*), and also replicated the *ZC3H11B* gene. Notably, five of these loci had been associated with refractive error in previous GWAS studies.

Several relatively small GWAS have been performed for corneal curvature; and identified associations with *FRAP1, PDGFRA* (also associated with eye size), *CMPK1,* and *RBP3* [94, 100–103]. More recently Miyake et al. published a two-stage GWAS for three myopia-related traits: axial length, corneal curvature, and refractive error [103, 104]. The study was performed in 9804 Japanese individuals, with trans-ethnic replication in Chinese and Caucasian individuals. A novel gene, *WNT7B*, was identified for axial length ($p = 3.9 \times 10^{-13}$) and corneal curvature ($p = 2.9 \times 10^{-40}$), while the previously reported association with *GJD2* and refractive error was replicated.

26.7.2 Genome-Wide Pathway Analyses

The main goal of GWAS is to improve insight on the molecules involved in disease and help identify disease mechanisms. For myopia, a retina-to-sclera signaling cascade had been proposed for many years (see accompanying paper IMI—Experimental models of emmetropization and myopia [105]), but knowledge on its molecular drivers was limited. Several attempts were made to translate the findings from refractive error GWAS into this cascade [88, 90, 106]. Here we provide an overview of genes annotated to the risk variants and their relationship to the underlying biological mechanism.

Deducted from the CREAM GWAS, pathways included neurotransmission (*GRIA4*), ion transport (*KCNQ5*), retinoic acid metabolism (*RDH5*), extracellular matrix remodeling (*LAMA2, BMP2*), and eye development (*SIX6, PRSS56*). Likewise, 23andMe proposed extracellular matrix remodeling (*LAMA2, ANTXR2*), the visual cycle (*RDH5, RGR, KCNQ5*), neuronal development (*KCNMA1, RBFOX1, LRRC4C, NGL-1, DLG2, TJP2*), eye and body growth (*PRSS56, BMP4, ZBTB38, DLX1*), and retinal ganglion cells (*ZIC2, SFRP1*) [107] as functions. Hysi et al. performed pathway analyses using both the CREAM and 23andMe GWAS, [108] and reported that plasma membrane, cell–cell adhesion, synaptic transmission, calcium ion binding, and cation channel activity were significantly overrepresented in refractive error in two British cohorts. Furthermore, by examining known protein–protein interactions, the investigators identified that many genes are related to cell cycle and growth pathways such as the MAPK and TGF-beta/SMAD pathways.

The latest update on pathway analysis in myopia stems from the meta-GWAS from CREAM and 23andMe [18]. TGF-beta signaling pathway was a key player; the association with the *DRD1* gene provided genetic evidence for a dopamine pathway. Most genes were known to play a role in the eye, [109] and most significant gene sets were "abnormal photoreceptor inner segment morphology" (Mammalian Phenotype Ontology (MP) 0003730; $P = 1.79 \times 10^{-7}$); "thin retinal outer nuclear layer" (MP 0008515); "detection of light stimulus" (Gene Ontology (GO) 0009583); "non-motile primary cilium" (GO 0031513); and "abnormal anterior-eye-segment morphology" (MP 0005193). Notably, *RGR, RP1L1, RORB,* and *GNB3* were present in all of these meta-gene sets. Taken together, retinal cell physiology and light processing are clearly prominent mechanisms for refractive error development, and all cell types of the neurosensory retina, retinal pigment epithelium, vascular endothelium, and extracellular matrix appear to be involved (Fig. 26.3). Novel mechanisms included rod-and-cone bipolar synaptic neurotransmission, anterior-segment morphology, and angiogenesis [18]. At the time of publication of this book chapter, Hysi et al. just published the largest meta-GWAS to date identifying 336 new genetic loci associated with refractive error [110]. This study revealed additional path-

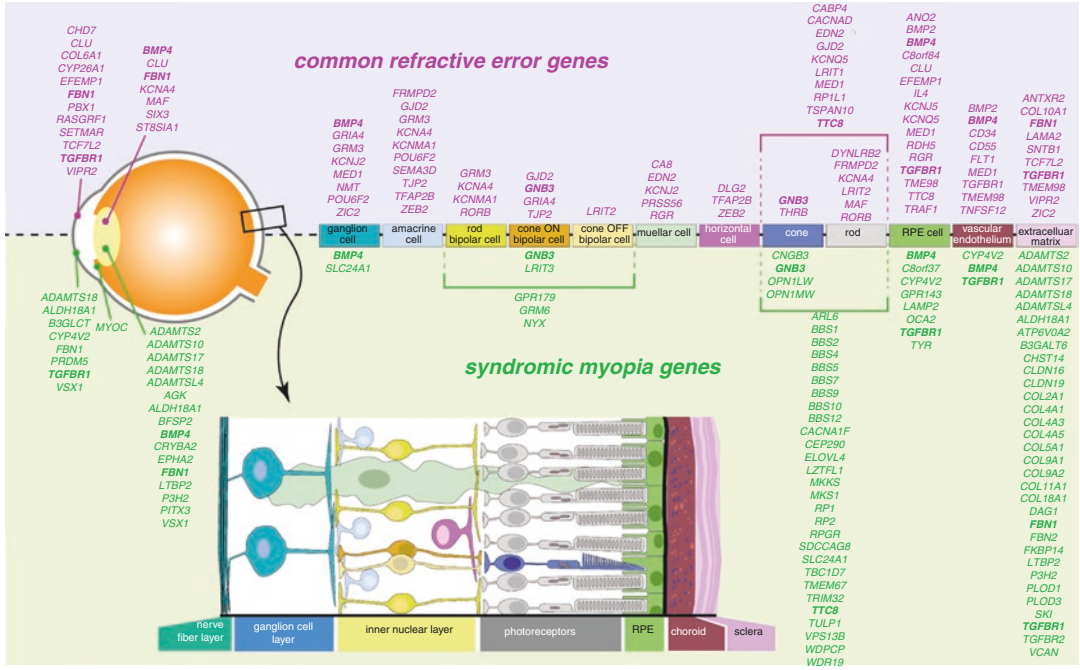

Fig. 26.3 Expression in retina of common refractive error genes and syndromic myopia genes. Schematic overview of expression in retinal cells of refractive error and syndromic myopia genes according to literature. Bold: genes identified for both common refractive error and in syndromic myopia

ways including circadian rhythm and pigmentation. These results weren't thoroughly incorporated in this chapter.

26.7.3 Astigmatism

A pioneering GWAS study for corneal astigmatism in 4254 participants identified a genome-wide significant locus within the promoter region of the *PDGFRA* gene on chromosome 4q12 [111]. This locus was subsequently replicated by a much larger CREAM consortium study of 31,370 participants, which demonstrated that the locus was associated with corneal astigmatism in both European and Asian individuals [112]. The *NRXN1* gene was discovered in a CREAM GWAS for *refractive* astigmatism, which analyzed a total of 45,931 individuals. Recently, researchers from the UK Biobank Eye and Vision Consortium identified association of the *ZC3H11B*, *LINC00340*, *HERC2/OCA2,* and *NPLOC4/TSPAN10* genes with corneal astigmatism, with a high degree of overlap between loci associated with corneal and refractive astigmatism (genetic correlation = 0.85) [113]. In contrast to findings from twin and family studies, the SNP heritability of astigmatism traits has been estimated to be <10% [112, 113]. The reason for the low SNP heritability for astigmatism and the paucity of genome-wide significant GWAS loci, in comparison to findings for spherical refractive error, is not clear. However, a greater role for rare variants in susceptibility to astigmatism is one potential explanation.

26.8 Whole-Exome and Whole-Genome Sequencing

Unlike GWAS, whole-exome sequencing (WES) and whole-genome sequencing (WGS) have the potential to investigate rare variants. Exomes are interesting as they directly contribute to protein translation, but they constitute only ~1% of the entire genome. WGS allows for identification of variants across the entire genome, but requires a high-throughput computational infrastructure and remains costly.

WES has been conducted primarily in case–control studies of early-onset high myopia or in specific families with a particular phenotype (i.e., myopic anisometropia) or inheritance pattern (i.e., X-linked) [114–117]. Several novel mutations in known myopia genes were identified this way: *CCDC111*, [115] *NDUFAF7*, [116] *P4HA2*, [114] *SCO2*, [118] *UNC5D*, [117] *BSG*, [119] *ARR3*, [120] *LOXL3*, [121] *SLC39A5*, [122] *LRPAP1*, [123] *CTSH*, [123] *ZNF644* [124, 125]. Although most genetic variants displayed an autosomal-dominant hereditary pattern, [114, 118, 124, 125] X-linked heterozygous mutations were identified in *ARR3,* only in female family members [120]. The functions of these novel genes include DNA transcription (*CCDC111, ZNF644*), mitochondrial function (*NDUFAF7, SCO2)*, collagen synthesis (*P4HA2*), cell signaling (*UNC5D, BSG*), retina-specific signal transduction (*ARR3*), transforming-growth factor-beta pathway (*LOXL3, SLC39A5, LRPAP1*), and degradation of proteins in lysosomes (*CTSH*). Jiang et al. investigated family members with high myopia and identified new mutations in *LRPAP1 (LDL Receptor Related Protein Associated Protein 1), CTSH (cathepsin H), ZNF644 (zinc finger protein 644 isoform 1), SLC39A5* (solute carrier family 39 (metal ion transporter), member 5), and *SCO2 (SCO2, cytochrome c oxidase assembly protein)* [125].

Many clinicians have noticed that retinal dystrophies and ocular developmental disorders often coincide with myopia [121]. This triggered Sun et al. to evaluate variants in a large number of retinal dystrophy genes in early-onset high myopia in 298 unrelated myopia probands and their families, and they thereby identified 29 potentially pathogenic mutations in *COL2A1, COL11A1, PRPH2, FBN1, GNAT1, OPA1, PAX2, GUCY2D, TSPAN12, CACNA1F,* and *RPGR* and most had an autosomal-dominant inheritance pattern [126]. Kloss et al. performed WES in 14 families with high myopia and identified 104 new genetic variants located in both known MYP loci (e.g., *AGRN, EME1*, and *HOXA2*) as well as in new loci (e.g., *ATL3* and *AKAP12*) [127].

To date, WGS has not been conducted for myopia or refractive error, most likely due to the reasons mentioned above. When costs for WGS decrease, these studies will undoubtedly be conceived. A WGS study on height, a trait comparable to myopia with heritability estimates around 80%, revealed two new parental imprinting regions affecting growth regulation [128].

26.9 Gene–Environment Interaction

It has become clear that environmental factors are driving the recent epidemic rise in the prevalence of myopia [129–133]. To date, the most influential and consistent environmental factor is education. Studies have estimated that individuals going onto higher education have double the myopia prevalence compared to those who leave school after only primary education [134–136]. Education has been a primary focus for gene–environment (GxE) interaction analyses in myopia. GxE studies have the potential to show modification of the effect of risk variants by environmental exposures but can also reveal genetic associations that were hidden in unexposed individuals.

One of the first GxE studies for myopia investigated variants in matrix metalloproteinases genes (*MMP1-MMP10*). Two SNPs (rs1939008 and rs9928731) that were first found to be associated with refraction in Amish families were also associated in a lower but not in the higher education group of the Age-Related Eye Disease Study (AREDS) study. These results suggest that variants in these genes may play a role in refractive variation in individuals not exposed to myopic triggers [61, 137]. In contrast, a study combining human GWAS data and animal models of myopia provided an experimental example of GxE interaction involving a rare variant in the *APLP2*-gene only in children exposed to large amounts of daily reading [138]. Additionally, an analysis performed in five Singapore cohorts found risk variants in *DNAH9, GJD2,* and *ZMAT4 were* more strongly associated in individuals who achieved higher secondary or university education [139]. Significant biological interaction between education and other risk variants was studied using a genetic risk score of all known risk variants at the time ($n = 26$) derived from the

CREAM meta-GWAS [140]. European subjects with a high genetic load in combination with university-level education had a far greater risk of myopia than those with only one of these two factors. A study investigating GxE interactions in children and the major environmental risk factors, nearwork, time outdoors and 39 SNPs derived from the CREAM meta-GWAS revealed nominal evidence of interaction with nearwork (top variant in *ZMAT4*) [140, 141].

Genome-wide interaction studies (GEWIS) using all variants from the CREAM meta-GWAS revealed three novel loci (*AREG, GABRR1,* and *PDE10A*) for GxE in Asian populations, whereas no interaction effects were observed in Europeans due to many reasons, such as the quantitative differences in the intensity of near work during childhood [50]. Up to now, there is no robust evidence that there are fundamental differences in the genetic background of myopia risk between Europeans and Asians.

26.10 Mendelian Randomization

Mendelian randomization (MR) is a method that allows one to test or estimate a causal effect from observational data in the presence of confounding factors. MR is a specific type of instrumental variable analysis that uses genetic variants with well-understood effects on exposures or modifiable biomarkers [142, 143]. Importantly, the SNPs chosen as instrumental variables must only affect the disease status via their effect on the exposure of interest, not by directly influencing the disease status [144]. MR is particularly valuable in situations where randomized controlled trials are not feasible, where it is applied to help elucidate biological pathways.

Currently, three studies have been published on MR in refractive error and myopia. The first, published in 2016, explored the effect of education on myopia [145]. This study constructed polygenic risk scores of genetic variants found in GWAS for educational attainment and used these as the instrumental variable. Subsequently, results of three cohorts (KORA, AREDS, BMES; total $N = 5649$) were meta-analyzed. Strikingly, ~2 years of education was associated with a myopic shift of -0.92 ± 0.29 diopters ($P = 1.04 \times 10^{-3}$), which was even larger than the observed estimate. Similar results were observed in data from the UK Biobank study ($N = 67{,}798$); MR was performed and the causality of education was tested for myopic refractive error bi-directionally [146]. Genetic variants for years of education from Social Science Genetic Association Consortium (SSGAC) and 23andMe studies were considered. Analyses of the observational data suggested that every additional year of education was associated with a myopic shift of −0.18 dioptres (D)/year (95% CI −0.19 to −0.17; $P < 2.0^{-16}$). MR suggested the true causal effect was stronger: −0.27 D/year (−0.37 to −0.17; $P = 4.0^{-8}$). Notably, there was no evidence that myopia was a cause for spending more years in education ($P = 0.6$). The conclusion from these studies was that education appears truly causally related to myopia, and effects calculated by the current observational studies may even be underestimated.

Because several studies had proposed that vitamin D has a protective effect against myopia [147–149], the third MR study investigated the causality of low vitamin D concentrations on myopia. Genetic variants of the *DHCR7, CYP2R1, GC,* and *CYP24A1* genes with known effects on serum levels of vitamin D were used as instrumental variables in a meta-analysis of refractive error in CREAM ($N_{EUR} = 37{,}382$ and $N_{ASN} = 8376$). The estimated effects of vitamin D on refractive error were small in both ethnicities (Caucasians: −0.02 [95% CI −0.09, 0.04] D per 10 nmol/l increase in vitamin D concentration; Asians: 0.01 [95% CI −0.17, 0.19] D per 10 nmol/l increase). These results suggest that the causal effect of vitamin D on myopia is very small, if any. Therefore, higher vitamin D levels are unlikely to represent the causal mechanism by which time spent outdoors protects against incident myopia.

26.11 Epigenetics

Epigenetic changes refer to functionally relevant changes to the genome that do not involve the nucleotide sequence of DNA. They represent other changes of the helix structure, such as DNA methylation and histone modification, [150] and these changes can regulate gene expression. Non-coding RNAs are small molecules that can also regulate gene expression, mainly at the posttranscriptional level; they can be epigenetically controlled but can also drive modulation of the DNA chromatin structure themselves [151]. Investigations into epigenetic changes of eye diseases still face some important technological hurdles. High-throughput next-generation sequencing technologies and high-resolution genome-wide epigenetic profiling platforms are still under development, and accessibility of RNA expression in human ocular tissues [152] is limited. Moreover, epigenetic changes are tissue and time-specific, so it is essential to study the right tissue at the correct developmental stage. Animal models are often used as a first step before moving to humans, although epigenetic processes are not always conserved across species. Nevertheless, there have been some attempts to reveal epigenetic changes involved in myopia development.

An experiment using monocular form deprivation in a mouse model found that hypermethylation of CpG sites in the promoter/exon 1 of *COL1A1* may underlie reduced collagen synthesis at the transcriptional level in myopic scleras [153]. A human study analyzing myopes found that methylation of the CpG sites of the CRYAA promotor leads to lower expression of *CRYAA* in human lens epithelial cells [154].

Myopia studies evaluating the role of non-coding RNAs are more common. The latest GWAS meta-analysis found 31 loci residing in or near regions transcribing small non-coding RNAs, thus hinting toward the key role of post-transcriptional processes and epigenetic regulation [18, 151]. MicroRNAs, or miRNAs, are the best-characterized family of small non-coding RNAs. In their mature form, they are approximately 19–24 nucleotides in length and regulate hundreds of genes. They are able to bind to 3′ UTR regions on RNA polymers by sequence-specific posttranscriptional gene silencing; one miRNA can regulate the translation of many genes. MiRNAs have been a hot topic in the last years due to the potential clinical application of these small RNA sequences: accessibility of the retina for miRNA-based therapeutic delivery has great potential for preventing and treating retinal pathology [155]. In a case–control study, Liang et al. [156] identified a genetic variant, rs662702, that was associated with the risk of extreme myopia in a Taiwanese population. The genetic variant was located at the 3′-UTR of PAX6, which is decreased in myopia. rs662702 is localized near the seed region of miR-328, and the C > T substitution leads to a mismatch between miR-328 and PAX6 mRNA. Further functional study indicated that the risk C allele reduced PAX6 expression relative to the T allele, which could result from the knockdown effect of the C allele by miR-328. Therefore, reducing miR-328 may be a potential strategy for preventing or treating myopia [63]. Another study focused on miR-184. This miRNA is the most abundant one in the cornea and the crystalline lens, and sequence mutations have been associated with severe keratoconus with early-onset anterior polar cataract. Lechner et al. [156, 157] sequenced miR-184 in 96 unrelated Han southern Chinese patients with axial myopia, but no mutations were detected. Xie et al. [158] analyzed rs157907 A/G in miR-29a and rs10877885 C/T in let-7i in a severe myopia case–control study (N_{cases} = 254; $N_{controls}$ = 300). The G allele of the rs157907 locus was significantly associated with decreased risk of severe myopia (P = 0.04), launching the hypothesis that rs157907 A/G might regulate miR-29a expression levels. Functional studies are needed to provide evidence for this theory.

26.12 Concluding Remarks

Since myopia is becoming a global epidemic, research unraveling the underlying genetic pathophysiology has increased in the last decade. The CREAM consortium enabled large genome-wide

studies and played a pioneering role in identifying new, mostly common, myopia genes. Future research should focus on finding rare variants with high impact and on integration of all levels of results, including genetic, epigenetic, and environmental findings. This can be done by setting up even larger collaborations and using large data sets and next-generation sequencing techniques. Ultimately, this may lead to improved identification of high-risk myopes, better treatment of progressive myopia, and its complications which in the longer term will reduce the visual burden of myopia.

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CREAM Membership List

The CREAM Consortium

Joan E. Bailey-Wilson[1], Paul Nigel Baird[2], Amutha Barathi Veluchamy[3-5], Ginevra Biino[6], Kathryn P. Burdon[7], Harry Campbell[8], Li Jia Chen[9], Ching-Yu Cheng[10-12], Emily Y. Chew[13], Jamie E. Craig[14], Phillippa M. Cumberland[15], Margaret M. Deangelis[16], Cécile Delcourt[17], Xiaohu Ding[18], Cornelia M. van Duijn[19], David M. Evans[20-22], Qiao Fan[23], Maurizio Fossarello[24], Paul J. Foster[25], Puya Gharahkhani[26], Adriana I. Iglesias[19,27,28], Jeremy A. Guggenheim[29], Xiaobo Guo[18,30], Annechien E.G. Haarman[19,28], Toomas Haller[31], Christopher J. Hammond[32], Xikun Han[26], Caroline Hayward[33], Mingguang He[2,18], Alex W. Hewitt[2,7,34], Quan Hoang[3,35], Pirro G. Hysi[32], Robert P. Igo Jr.[36], Sudha K. Iyengar[36-38], Jost B. Jonas[39,40], Mika Kähönen[41,42], Jaakko Kaprio[43,44], Anthony P. Khawaja[25,45], Caroline C. W. Klaver[19,28,46], Barbara E. Klein[47], Ronald Klein[47], Jonathan H. Lass[36,37], Kris Lee[47], Terho Lehtimäki[48,49], Deyana Lewis[1], Qing Li[50], Shi-Ming Li[40], Leo-Pekka Lyytikäinen[48,49], Stuart MacGregor[26], David A. Mackey[2,7,34], Nicholas G. Martin[51], Akira Meguro[52], Andres Metspalu[31], Candace Middlebrooks[1], Masahiro Miyake[53], Nobuhisa Mizuki[52], Anthony Musolf[1], Stefan Nickels[54], Konrad Oexle[55], Chi Pui Pang[9], Olavi Pärssinen[56,57], Andrew D. Paterson[58], Norbert Pfeiffer[54], Ozren Polasek[59,60], Jugnoo S. Rahi[15,25,61], Olli Raitakari[62,63], Igor Rudan[8], Srujana Sahebjada[2], Seang-Mei Saw[64,65], Dwight Stambolian[66], Claire L. Simpson[1,67], E-Shyong Tai[65], Milly S. Tedja[19,28], J. Willem L. Tideman[19,28], Akitaka Tsujikawa[53], Virginie J.M. Verhoeven[19,27,28], Veronique Vitart[33], Ningli Wang[40], Juho Wedenoja[43,68], Wen Bin Wei[69], Cathy Williams[22], Katie M. Williams[32], James F. Wilson[8,33], Robert Wojciechowski[1,70,71], Ya Xing Wang[40], Kenji Yamashiro[72], Jason C. S. Yam[9], Maurice K.H. Yap[73], Seyhan Yazar[34], Shea Ping Yip[74], Terri L. Young[47], Xiangtian Zhou[75]

Affiliations

1. Computational and Statistical Genomics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland, USA.
2. Centre for Eye Research Australia, Ophthalmology, Department of Surgery, University of Melbourne, Royal Victorian Eye and Ear Hospital, Melbourne, Australia.
3. Singapore Eye Research Institute, Singapore National Eye Centre, Singapore.
4. Duke-NUS Medical School, Singapore, Singapore.
5. Department of Ophthalmology, National University Health Systems, National University of Singapore, Singapore.
6. Institute of Molecular Genetics, National Research Council of Italy, Pavia, Italy.
7. Department of Ophthalmology, Menzies Institute of Medical Research, University of Tasmania, Hobart, Australia.
8. Centre for Global Health Research, Usher Institute for Population Health Sciences and Informatics, University of Edinburgh, Edinburgh, UK.
9. Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong Eye Hospital, Kowloon, Hong Kong.
10. Department of Ophthalmology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore.

11. Ocular Epidemiology Research Group, Singapore Eye Research Institute, Singapore National Eye Centre, Singapore.
12. Ophthalmology and Visual Sciences Academic Clinical Program (Eye ACP), Duke-NUS Medical School, Singapore.
13. Division of Epidemiology and Clinical Applications, National Eye Institute/ National Institutes of Health, Bethesda, USA.
14. Department of Ophthalmology, Flinders University, Adelaide, Australia.
15. Great Ormond Street Institute of Child Health, University College London, London, UK.
16. Department of Ophthalmology and Visual Sciences, John Moran Eye Center, University of Utah, Salt Lake City, Utah, USA.
17. Université de Bordeaux, Inserm, Bordeaux Population Health Research Center, team LEHA, UMR 1219, F-33000 Bordeaux, France.
18. State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China.
19. Department of Epidemiology, Erasmus Medical Center, Rotterdam, The Netherlands.
20. Translational Research Institute, University of Queensland Diamantina Institute, Brisbane, Queensland, Australia.
21. MRC Integrative Epidemiology Unit, University of Bristol, Bristol, UK.
22. Department of Population Health Sciences, Bristol Medical School, Bristol, UK.
23. Centre for Quantitative Medicine, DUKE-National University of Singapore, Singapore.
24. University Hospital 'San Giovanni di Dio', Cagliari, Italy.
25. NIHR Biomedical Research Centre, Moorfields Eye Hospital NHS Foundation Trust and UCL Institute of Ophthalmology, London, UK.
26. Statistical Genetics, QIMR Berghofer Medical Research Institute, Brisbane, Australia.
27. Department of Clinical Genetics, Erasmus Medical Center, Rotterdam, The Netherlands.
28. Department of Ophthalmology, Erasmus Medical Center, Rotterdam, The Netherlands.
29. School of Optometry and Vision Sciences, Cardiff University, Cardiff, UK.
30. Department of Statistical Science, School of Mathematics, Sun Yat-Sen University, Guangzhou, China.
31. Institute of Genomics, University of Tartu, Tartu, Estonia.
32. Section of Academic Ophthalmology, School of Life Course Sciences, King's College London, London, UK.
33. MRC Human Genetics Unit, MRC Institute of Genetics and Molecular Medicine, University of Edinburgh, Edinburgh, UK.
34. Centre for Ophthalmology and Visual Science, Lions Eye Institute, University of Western Australia, Perth, Australia.
35. Department of Ophthalmology, Columbia University, New York, USA.
36. Department of Population and Quantitative Health Sciences, Case Western Reserve University, Cleveland, Ohio, USA.
37. Department of Ophthalmology and Visual Sciences, Case Western Reserve University and University Hospitals Eye Institute, Cleveland, Ohio, USA.
38. Department of Genetics, Case Western Reserve University, Cleveland, Ohio, USA.
39. Department of Ophthalmology, Medical Faculty Mannheim of the Ruprecht-Karls-University of Heidelberg, Mannheim, Germany.
40. Beijing Tongren Eye Center, Beijing Tongren Hospital, Beijing Institute of Ophthalmology, Beijing Key Laboratory of Ophthalmology and Visual Sciences, Capital Medical University, Beijing, China.
41. Department of Clinical Physiology, Tampere University Hospital and School of Medicine, University of Tampere, Tampere, Finland.
42. Finnish Cardiovascular Research Center, Faculty of Medicine and Life Sciences, University of Tampere, Tampere, Finland.
43. Department of Public Health, University of Helsinki, Helsinki, Finland.

44. Institute for Molecular Medicine Finland FIMM, HiLIFE Unit, University of Helsinki, Helsinki, Finland.
45. Department of Public Health and Primary Care, University of Cambridge, Cambridge, UK.
46. Department of Ophthalmology, Radboud University Medical Center, Nijmegen, The Netherlands.
47. Department of Ophthalmology and Visual Sciences, University of Wisconsin–Madison, Madison, Wisconsin, USA.
48. Department of Clinical Chemistry, Finnish Cardiovascular Research Center-Tampere, Faculty of Medicine and Life Sciences, University of Tampere.
49. Department of Clinical Chemistry, Fimlab Laboratories, University of Tampere, Tampere, Finland.
50. National Human Genome Research Institute, National Institutes of Health, Baltimore, USA.
51. Genetic Epidemiology, QIMR Berghofer Medical Research Institute, Brisbane, Australia.
52. Department of Ophthalmology, Yokohama City University School of Medicine, Yokohama, Kanagawa, Japan.
53. Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan.
54. Department of Ophthalmology, University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany.
55. Institute of Neurogenomics, Helmholtz Zentrum München, German Research Centre for Environmental Health, Neuherberg, Germany.
56. Department of Ophthalmology, Central Hospital of Central Finland, Jyväskylä, Finland.
57. Gerontology Research Center, Faculty of Sport and Health Sciences, University of Jyväskylä, Jyväskylä, Finland.
58. Program in Genetics and Genome Biology, Hospital for Sick Children and University of Toronto, Toronto, Ontario, Canada.
59. Gen-info Ltd, Zagreb, Croatia.
60. University of Split School of Medicine, Soltanska 2, Split, Croatia.
61. Ulverscroft Vision Research Group, University College London, London, UK.
62. Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, Turku, Finland.
63. Department of Clinical Physiology and Nuclear Medicine, Turku University Hospital, Turku, Finland.
64. Myopia Research Group, Singapore Eye Research Institute, Singapore National Eye Centre, Singapore.
65. Saw Swee Hock School of Public Health, National University Health Systems, National University of Singapore, Singapore.
66. Department of Ophthalmology, University of Pennsylvania, Philadelphia, Pennsylvania, USA.
67. Department of Genetics, Genomics and Informatics, University of Tennessee Health Sciences Center, Memphis, Tennessee.
68. Department of Ophthalmology, University of Helsinki and Helsinki University Hospital, Helsinki, Finland.
69. Beijing Tongren Eye Center, Beijing Key Laboratory of Intraocular Tumor Diagnosis and Treatment, Beijing Ophthalmology and Visual Sciences Key Lab, Beijing Tongren Hospital, Capital Medical University, Beijing, China.
70. Department of Epidemiology and Medicine, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA.
71. Wilmer Eye Institute, Johns Hopkins Medical Institutions, Baltimore, Maryland, USA.
72. Department of Ophthalmology, Otsu Red Cross Hospital, Nagara, Japan.
73. Centre for Myopia Research, School of Optometry, The Hong Kong Polytechnic University, Hong Kong, Hong Kong.
74. Department of Health Technology and Informatics, The Hong Kong Polytechnic University, Hong Kong, Hong Kong.
75. School of Ophthalmology and Optometry, Eye Hospital, Wenzhou Medical University, China.

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27 Oncologic Properties of Retinoblastoma Genes

Wai Kit Chu, Jason C. S. Yam, Li Jia Chen, and Chi Pui Pang

Abstract

Retinoblastoma is the commonest pediatric intraocular cancer across various populations. Following the "two-hit model," both alleles are inactivated at the disease-causing gene, *RB1*. RB has been well studied to establish its "classical" function in regulating cell cycle progression and the transcription machinery. Recent studies have identified additional genetic factors contributing to retinoblastoma tumor development. Some of these newly identified genetic factors, including MGMT and MLH1, are well known for their roles in maintaining genome stability. On the other hand, novel functions have also been found in RB in preserving genome stability. As genome instability is a major driving force of cancer, understanding the oncologic properties of RB and other retinoblastoma related genes could improve our knowledge and disease management in retinoblastoma and other RB mutated cancers.

Keywords

Retinoblastoma · RB · RASSF1A · MGMT MLH1 · Genome stability

W. K. Chu (✉) · J. C. S. Yam · L. J. Chen · C. P. Pang
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, Hong Kong
e-mail: waikit@cuhk.edu.hk

Retinoblastoma is the most common intraocular cancer in children worldwide. Loss-of-function mutations in both alleles of the *RB1* gene are needed for the tumorigenesis of retinoblastoma. The *RB1* gene was the first tumor suppressor gene that was successfully cloned [1]. Subsequent studies identified the "classical" function of the gene product of *RB1*, RB, which acts as a signal transducer connecting the cell cycle progression with the transcription machinery [2]. Other extensive studies on the *RB1* gene identified additional non-canonical functions [3]. Apart from *RB1*, other genetic alterations also contribute to the retinoblastoma tumor development. Studies on the *RB1* gene and other retinoblastoma related genes have contributed significantly to the understanding of the disease mechanism. In this chapter, we summarize ours and others recent findings about the oncologic properties of retinoblastoma genes.

27.1 *RB1* Inactivation in Hong Kong Chinese Retinoblastoma Patients

By examining the promoter and the coding sequence of *RB1* from 42 Chinese sporadic retinoblastoma patients, 15 *RB1* mutations were identified in 38% (16/42) patients [4]. 19% (8/42) of these patients carried the *RB1* germline mutations. Out of these *RB1* mutations, 9 had not been reported before: E54X, S114X,

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I126S, g73779insG, D718N, IVS2 + 1G > C, IVS14 + 1G > C, IVS21 + 1G > C, and a complex alteration g78177G > T/g78176insTT leading to 543X [4]. One of these mutations, D718N, situates within the pocket B domain, which is important to regulate interaction with other proteins including E2F [5]. Three of these mutations, IVS2 + 1G > C, IVS14 + 1G > C, and IVS21 + 1G > C, alter the splice donor sequences that possibly lead to aberrant splicing. In addition to the DNA sequence alternation, we also investigated the methylation status of the CpG islands at the 5′ region of the *RB1* gene [4]. In all the 42 samples, no aberrant methylation in the *RB1* promoter could be detected, which was different from another reported study that 9.3% unilateral sporadic retinoblastoma cases and 1% bilateral or hereditary tumors showed hypermethylation [6]. In our study, both normal and cancerous retinoblastoma tissues were available in 15 cases. By analyzing the microsatellite markers located within or adjacent to the *RB1* gene on chromosome 13q14, 60% (9/15) of these samples showed loss of heterozygosity (LOH), which was similar to other reports [7–9]. Our results suggested loss-of-function mutations and LOH are the major mechanisms to inactivate *RB1* in sporadic retinoblastoma.

27.2 *RASSF1A* Inactivation in Hong Kong Chinese Retinoblastoma Patients

Early studies in childhood tumors frequently found methylation in the promoter region of *RASSF1A* (Ras association domain family 1, isoform A) [10]. RASSF1A is a tumor suppressor that can induce cell cycle arrest and inhibit the accumulation of the Cyclin D1, a protein that binds to the cyclin-dependent kinase 4 to phosphorylate RB [11]. We compared the *RASSF1A* promotor methylation status in micro-dissected tumor and normal retina tissues from 68 Hong Kong Chinese retinoblastoma patients and found that 82% of the tumor samples, but not in the adjacent normal retinal cells, showed hypermethylation in the *RASSF1A* promotor [12]. No mutation was detected in the *RASSF1A* coding region. In these hypermethylated samples, no *RASSF1A* transcripts could be detected [13]. We also detected *RASSF1A* promotor hypermethylation in two established retinoblastoma cell lines, Y79 and WERI-Rb1. Furthermore, in the WERI-Rb1 cells, 5-AzaCdR demethylation treatment could rescue the expression of *RASSF1A* and induce a small G2/M cell cycle arrest [12]. These results demonstrated the association of *RASSF1A* promotor hypermethylation with retinoblastoma.

27.3 *MGMT* Inactivation in Hong Kong Chinese Retinoblastoma Patients

Similar to the *RASSF1A*, promotor methylation was also commonly found in *MGMT* (O^6-methylguanine DNA methyltransferase) in childhood tumors [10]. MGMT is an important protein in preventing DNA mismatch mutation by removing a methyl group from the sixth position of guanine [14]. Hypermethylation of the *MGMT* promoter could be detected in 15% (15/68) of the Hong Kong Chinese retinoblastoma samples, but not in the Y79 and WERI-Rb1 cell lines [12]. Interestingly, we found that the absence of *MGMT* promotor hypermethylation was significantly associated with retinoblastoma at the advanced Reese-Ellsworth tumor stage ($P = 0.002$) [12]. These results demonstrated that the *MGMT* promotor hypermethylation could be useful for retinoblastoma prognosis.

27.4 *MLH1* Inactivation in Hong Kong Chinese Retinoblastoma Patients

Similar promotor hypermethylation was also commonly observed in another DNA mismatch gene *MLH1* in cancer [15, 16]. In particular, defective MLH1 is associated with microsatellite instability (MSI) affecting the repetitive microsatellite sequences in the global genome, which is associated with tumor development [17]. We detected *MLH1* promoter hypermethylation in

67% (34/51) of the Hong Kong Chinese retinoblastoma samples and the WERI-Rb1 cell line, but not in the Y79 cell line [18]. Hypermethylation in the *MLH1* promoter was associated with undetectable MLH1 protein expression. In 26 of these retinoblastoma samples, high and low high-frequency MSI could each be detected in 19% of them [18]. *MLH1* promoter methylation was significantly associated with retinoblastoma with well-differentiated histology ($P < 0.05$) [18]. However, no association could be detected between MSI status and the clinical and pathological features of retinoblastoma [18].

27.5 Novel Functions of RB in Genome Stability

Our works on *MGMT* and *MLH1* inactivation in retinoblastoma suggest that genome instability may be a major driving force of the oncogenesis in retinoblastoma. Recently, novel functions of RB have been reported in maintaining genome stability [19, 20]. For example, in RB knocked down cells, missegregated chromosomes could be observed frequently in mitotic cells [21, 22]. More broken DNA was also observed in RB inactivated cells [19]. These broken DNA also remained unrepaired for a longer time in RB depleted cells [23]. Importantly, RB depletion led to reduced cell survival rates in response to DNA double-strand break (DSB) inducing compounds including topoisomerase poisons etoposide and camptothecin [24]. There are two major mechanisms for the repair of DSBs: canonical nonhomologous end-joining (c-NHEJ) and homologous recombination (HR) [25]. c-NHEJ ligates DSBs by using little or no sequence homology at the DNA breakpoint, while HR repairs DSBs by using the sister chromatid or homologous chromosome as the template [26–28]. c-NHEJ is a relatively error-prone repair pathway that functions throughout the cell cycle, as compared to HR, which is a more precise repair pathway generating during S phase to the G2 phase of the cell cycle.

Recently RB was found to regulate both c-NHEJ and HR [24, 29, 30]. RB was reported to interact with XRCC5 (also named Ku80) and XRCC6 (also named Ku70), two key proteins binding to the DNA end, to promote c-NHEJ [29]. Apart from c-NHEJ, RB was also reported to promote HR by recruiting another protein BRG1 to DSBs [24]. BRG1 belongs to the SWI/SNF family of ATPases, which are able to remodel chromatin and is important for the HR pathway. These findings suggested that RB mutations would lead to compromised genome stability, which could potentially drive oncogenesis in retinoblastoma.

In addition to DNA double-strand break repair, RB was also involved in maintaining genome stability by enhancing chromosome cohesion [31]. Loss of RB altered the trimethylation of histone H4 on lysine 20 (H4K20me3), which disrupted the establishment of sister chromatid cohesion at centromeres during S phase. RB interacted directly with Suv4-20 h1 and Suv4-20 h2 to control H4K20me3, while this interaction was found to be independent to E2F [32, 33]. Compromised cohesion led to increased inter-kinetochore distance and reduced DNA replication fork elongation during S phase, elevated DNA damages, defective chromatid segregation in anaphase and chromosome copy number heterogeneity [31].

Apart from chromosome cohesion, RB was also reported to be involved in chromatin condensation [34]. RBF1, the RB homolog in *Drosophila*, interacted directly with CAP-D3, a component of the Condensin II complex, to promote CAP-D3 association with chromatin. Mutation in RBF1 led to extensive defects in chromatin condensation during mitosis. Importantly, the RB-CAP-D3 interaction could also be detected in human cells, suggesting RB may also regulate chromatin condensation in humans [34].

In addition to chromatin condensation, RB was reported to recruit another chromatin remodeling factor EZH2 to heterochromatin, genomic regions mainly composed of repetitive DNA sequences [35]. RB, together with E2F1, recruited EZH2 to diverse repeated sequences including simple repeats, satellites, long interspersed nuclear elements, endogenous retroviruses and transposon fragments [35]. The RB-E2F1-EZH2 complex maintained trimethylation of histone

H3 on lysine 27 (H3K27me3) to suppress the expression of these repeated sequences. Mice carrying Rb F832A mutation showed reduced EZH2 recruitment to the repeated sequences and less H3K27me3 at these repeated sequences. Importantly, these mutant mice showed a higher chance of getting lymphomas in the spleen and mesenteric lymph node [35]. In another study, RB was reported to interact with NuRD, a histone deacetylation enzyme, to suppress the expression of the long interspersed nuclear elements [36]. These results demonstrated RB is important to suppress expression at repeated sequences and tumor formation.

27.6 Conclusive Remarks

Our work in Hong Kong Chinese retinoblastoma patients identified important genetic factors of this disease. Interestingly, many of these genetic factors play important roles in preserving genome stability. Although the exact mechanism of how RB suppresses the retinoblastoma is not fully understood, the classical functions, as well as the recent novel discoveries of RB, have helped us to better understand the molecular pathogenesis of retinoblastoma and other RB mutated cancers.

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Oncologic Implications of Genetic and Epigenetic Basis of Pterygium

28

Wai Kit Chu, Chi Pui Pang, and Tsz Kin Ng

Abstract

Pterygium is a fast-growing hyperplastic growth from the conjunctiva over the limbus towards the central cornea. Although the exact pathological mechanism remains to be elucidated, it is strongly associated with the exposure to ultraviolet light. On the contrary, the contribution of cigarette smoking to the incidence of primary pterygium is inconsistent. Despite the fast-growing nature of pterygium, high expression of p53 has been consistently reported by multiple investigations. Recently, we reported the contribution of p53-binding protein MDM2 to the suppression of the p53-mediated apoptosis in human pterygium. Disruption of the MDM2-p53 interaction by Nutlin treatment showed specific killing of pterygium cells with low toxicity to conjunctiva cells. We also identified the involvement of the growth hormone-releasing hormone signaling pathway in the pathogenesis of pterygium. In addition, our recent meta-analysis identified a negative correlation of cigarette smoking with the occurrence of pterygium in current smokers. Major components of the cigarette smoke, including nicotine and cotinine, suppressed primary pterygium cell proliferation and migration, possibly through extracellular matrix remodeling and epithelial-to-mesenchymal transition. Our results underline the genetic and epigenetic basis of pterygium, which broadens the understanding of the oncologic-like properties of pterygium.

Keywords

Pterygium · Conjunctiva · p53 · MDM2 Nutlin · GHRH · Cigarette smoking · Nicotine

W. K. Chu
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, China
e-mail: waikit@cuhk.edu.hk

C. P. Pang
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, China

Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong, Shantou, Guangdong, China

T. K. Ng (✉)
Department of Ophthalmology and Visual Sciences, The Chinese University of Hong Kong, Hong Kong, China

Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong, Shantou, Guangdong, China

Shantou University Medical College, Shantou, Guangdong, China

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28.1 Oncologic Implications of Genetic Basis of Pterygium

28.1.1 p53 and MDM2 Expression in Pterygium

Pterygium is a triangular-shaped hyperplastic growth of the bulbar conjunctiva over the limbus. It has been reported that the fast-growing properties of pterygium are correlated with the expression of several proteins that are related to cancer, including cyclin D1, p27, p53, Bcl-2, Ki-67, and proliferating cell nuclear antigen (PCNA) [1, 2]. Among these proteins, there are multiple studies reported the elevated expression of p53 in pterygium [3–5]. The p53 protein has been shown to induce senescence and apoptosis to restrict the growth of cancer [6]. It seems contradictory for the fast-growing pterygium to have high p53 expression. Recently, we found that the mouse double minute 2 (MDM2) protein is also highly expressed in pterygium [7]. MDM2 is a p53 binding protein. It translocates p53 from the nucleus to cytoplasm and degrades p53 by ubiquitination [8]. We observed a high level of MDM2 in pterygium tissues and the MDM2 protein mainly localized to the nucleus [7]. Interestingly, we observed p53 concentrated in the cytoplasm. One of the p53 transcriptional regulated target genes, p21, was not expressed in pterygium tissues, suggesting the p53 transcriptional activity was not active in pterygium. Chemical antagonists including Nutlin have been developed to bind to the p53-binding pocket in MDM2 and to activate p53 [9]. We treated pterygium epithelial cells with Nutlin and found that the nuclear localization of p53, the expression of p21, and apoptosis could be induced [7, 10]. Our study demonstrated that, despite the high p53 expression in pterygium, it had a cytoplasmic localization in pterygium while MDM2 was mainly expressed in nuclei. Disruption of the MDM2-p53 interaction led to p53 localizing in the nucleus and p53 reactivation.

28.1.2 p53 and MDM2 Expression in Conjunctiva

Pterygium has been reported to be originated from conjunctiva [11]. In conjunctiva tissues isolated from pterygium patients, no p53 expression could be detected, and only very weak MDM2 expression was detected [7]. When we treated conjunctival cells with Nutlin up to 10 μM for 5 days, the condition leading to a significant reduction in viability and migration in pterygium cells, there was no significant change in the conjunctival cell survival and migration [10]. These results indicated that Nutlin had a targeted impact on pterygium cells but not conjunctival cells.

28.1.3 Nutlin as a Novel Treatment to Pterygium

Currently, intra-operative mitomycin C (MMC) treatment is commonly used to reduce the recurrence of pterygium [12, 13]. MMC is a natural anti-tumor antibiotic isolated from *Streptomyces caespitosus*. It is a DNA cross-linker, which can generate cross-links on the same DNA strand (intra-strand) and between DNA strands (inter-strand) [14]. We found that MMC caused a significant reduction in cell viability and migration in both conjunctiva and pterygium [10]. When we compared the effects of Nutlin and MMC treatments in the conjunctiva, we found that at the concentration of killing 50% of pterygium cells, 95% of conjunctival cells survived after Nutlin treatment. However, only 63% of conjunctival cells survived when treated with MMC at the concentration that killed half of the pterygium cells [10]. Our results showed that Nutlin could be more specific, compared to MMC, in killing pterygium cells with less effects on conjunctiva cells.

28.1.4 Growth Hormone-Releasing Hormone Signaling Pathway in Pterygium

Growth hormone-releasing hormone (GHRH) is a hormone synthesized in the hypothalamus. GHRH could bind to its receptor to induce synthesis and secretion of the growth hormone (GH) in the anterior pituitary [15]. Our recent studies found that the GHRH signaling pathway also exists in the eyes, suggesting the involvement of this pathway in various eye diseases [16, 17]. In retinoblastoma cells, the p53 mediated apoptosis is suppressed by MDM2 [18], and we found that antagonists of the GHRH receptor (GHRH-R) could induce apoptosis in retinoblastoma cells [17]. As we mentioned above, the transcriptional activity of p53 is also suppressed by MDM2 in primary pterygium cells [7]. Therefore, we hypothesized that the GHRH-R antagonist could also induce apoptosis in pterygium epithelial cells. Our results indicated that the receptors of GHRH and GH were highly expressed in pterygium epithelial cells [19]. Higher levels of GHRH and GH were detected in pterygium compared to conjunctiva cells. GHRH-R antagonist treatment could induce apoptosis in pterygium epithelial cells, which associated with elevated caspase 3 and reduced ERK1 expression, proteins that are important for the apoptosis and cell survival respectively [19]. Our findings indicated the important roles of the GHRH pathway in pterygium, suggesting GHRH-R antagonist could be developed as another novel treatment to pterygium.

28.2 Cigarette Smoking on Pterygium Development and Progression

Cigarette smoking is a modifiable risk factor for the development of multiple eye diseases, such as age-related macular degeneration [20, 21]. Yet, the contribution of cigarette smoking to the incidence of primary pterygium is inconsistent, as indicated by the epidemiological studies (Table 28.1). The Southern Harbin Eye Study found a significant association between cigarette smoking and pterygium in rural adult northern Chinese population with odds ratio (OR) of 1.90 [22]. In a dry and high-altitude province of Iran, cigarette smoking was significantly associated with a higher incidence of pterygium (OR = 5.46) [23]. In contrast, the Singapore Malay Eye Study reported that cigarette smoking is not associated with the incidence of pterygium in the adult Malay population but increased the odds of bilateral pterygium (OR = 1.50) [24]. Besides, a population-based survey of adult Latinos in Arizona (Proyecto VER) reported that current smokers were less likely to develop pterygium (OR = 0.75), compared to the pterygium patients who never smoke [25]. Furthermore, the Korean National Health and Nutrition Examination Survey demonstrated that the lifetime smokers have a reduced risk to develop pterygium (OR = 0.70) [26], whereas the Handan Eye Study in northern China showed that current smoking is a protective factor for pterygium (OR = 0.50) [27]. We previously conducted a systemic review and meta-analysis, and we found that cigarette smoking was associated with a reduced risk of pterygium in current smokers (OR = 0.68), but not in ex-smokers (OR = 1.05), and the association is independent of ultraviolet light exposure and gender [28]. Nevertheless, no mechanism between cigarette smoking and pterygium has been suggested [29–31]. The biological effects of cigarette smoking components on human primary pterygium cells remain elusive.

28.2.1 The Effect of Nicotine and Its Metabolites on Human Primary Pterygium Cells

Cigarette smoke contains over 4000 chemicals. Among these chemicals, nicotine is a key component and the determinant factor for addiction

Table 28.1 Epidemiological studies of cigarette smoking in primary pterygium

References	Year of publication	Population	Age	Sample size	Study design	Total pterygium patients	Smoker with pterygium (%)
McCarty CA et al	2000	Australia	40–101	5044	Population-based cross-sectional study	142	14.7
Saw SM et al	2000	Singapore	30+	186	Hospital-based case-control study	61	73.3
Wong TY et al	2000	Singapore	40–79	2000	Population-based cross-sectional study	120	/
Luthra R et al	2001	Barbado	40–84	2617	Population-based cross-sectional study	163	4.7
Gazzard G et al	2002	Indonesia	21+	1210	Population-based cross-sectional study	163	9.1
Al-Bdour MD et al	2004	Jordan	22–72	288	Hospital-based case-control study	96	15.3
Durkin SR et al	2007	Myanmar	40+	2076	Population-based cross-sectional study	163	31.9
Nemesure B et al	2008	Barbado	40–84	1888	Population-based study	218	10.6
Fotouhi A et al	2009	Iran	1+	4564	Population-based cross-sectional study	66	/
Shiroma H et al	2009	Japan	40+	3747	Population-based cross-sectional study	1154	39
West S et al	2009	America	40+	4767	Population-based cross-sectional study	772	53.8
Cajucom-Uy H et al	2010	Singapore	40–79	3280	Population-based cross-sectional study	508	49
Viso E et al	2011	Spain	40+	619	Population-based cross-sectional study	298	3.69
Asokan R et al	2012	India	40+	7774	Population-based cross-sectional study	740	/
Li Z et al	2012	China	50–96	5057	Population-based cross-sectional study	323	/
Marcus A et al	2012	Singapore	40+	8906	Population-based cross-sectional study	900	/
Rezvan F et al	2012	Iran	40–64	5190	Population-based cross-sectional study	489	/
Zhong H et al	2012	China	50–92	2133	Population-based cross-sectional study	832	22.4

Table 28.1 (continued)

References	Year of publication	Population	Age	Sample size	Study design	Total pterygium patients	Smoker with pterygium (%)
Lanping S et al	2013	China	40+	6599	Population-based cross-sectional study	401	/
Marmamula S et al	2013	India	30–102	5586	Population-based cross-sectional study	655	35.7
Nangia V et al	2013	India	30+	4711	Population-based cross-sectional study	608	/
Rim THT et al	2013	South Korea	30+	14,920	Population-based cross-sectional study	4307	/
Tano T et al	2013	Japan	40–74	2312	Population-based cross-sectional study	101	12.9
Zhao L et al	2013	China	40+	2695	Population-based study	129	31

to smoking [32]. Moreover, nicotine is also the major component in cigarette replacements, including nicotine patches and the recently popular electronic cigarettes. Nicotine binds to and activates the pentameric nicotinic acetylcholine receptors, composed of α, β, γ, δ, and ε subunits [33]. Our research group found that the α5, β1, and γ subunits of nicotinic acetylcholine receptors are the major components in human primary pterygium cells, whereas the α1, α6, α9, and β2 subunits are moderately expressed [34]. This indicates that human primary pterygium cells could be influenced by the exposure of nicotine.

Under physiological conditions, nicotine has a half-life of 2 h in the human body. It is continuously metabolized by hepatic cytochrome P450 enzyme CYP2A6 into cotinine [35], which is the major metabolite of nicotine. In contrast, cotinine has a half-life of 19 h [36]. Besides, the plasma level of cotinine (1.02–1.73 μM) in daily cigarette smokers is also higher than that of nicotine (0.08–0.15 μM) [37, 38], indicating that the higher concentration and duration of cotinine should have a potent effect on the biological activities.

The neoplastic-like properties of pterygium are determined by multiple factors. The abundance of pterygium cells along the hyperplastic process is determined by the proliferation of the pterygium cells. Our recent study demonstrated that upon continuous exposure of 0.15 μM nicotine and 2 μM cotinine treatment, the proliferation rate of human primary pterygium cells is significantly retarded by 16.04% [34]. However, the reduced pterygium cell proliferation under 0.15 μM nicotine-2 μM cotinine treatment was not due to cell apoptosis. Apart from cell proliferation, the movement of pterygium cells towards the central cornea is characterized by the migration ability of the pterygium cells. Nicotine-cotinine treatment significantly inhibited human primary pterygium cell migration by 11.93% [34]. Collectively, our study suggested that nicotine and cotinine could hinder human primary pterygium cell proliferation and migration properties *in vitro*.

28.2.2 The Mechanistic Regulations of Nicotine and Its Metabolites on Human Primary Pterygium Cells

The pathogenesis of pterygium remains elusive. Multiple signaling pathways have been suggested to be involved in pterygium development, including the nuclear factor kappa B (NF-κB) pathway [39], p38 MAPK pathway [40], and mTOR pathway [41]. The aberrant accumulation of extracellular matrix molecules and elastotic degeneration has been found in the stroma of pterygium tissues [42]. Coherently, we have reported that the involvement of matrix metalloproteinase-2 (MMP-2) and MMP-9 in the pathogenesis of pterygium [43]. Extracellular matrix (ECM) remodeling is believed to be involved in the progression of pterygium [44]. ECM proteins, regulated by metalloproteinases, can provide the structure and biochemical support for cell adhesion and migration [45]. The expression of different MMPs could be associated with the invasion and migration abilities of pterygium cells as well as the disease progression [46–49]. Based on the multiplex ELISA, we detected high expression of MMP-1, MMP-2, and MMP-3, but low expression of MMP-9, in human primary pterygium cells, whereas MMP-7 and MMP-13 expression were not detected [34]. Nicotine and cotinine treatments significantly reduce the MMP-1 and MMP-9 expression in human primary pterygium cells, indicating that the retarded cell proliferation and migration properties of human primary pterygium cells could be related to reduced expression of MMP-1 and MMP-9 proteins in pterygium cells. ECM remodeling could be a target influenced by nicotine and cotinine in human primary pterygium cells.

In addition to the ECM remodeling, the excessive proliferation of pterygium tissues could be related to the aberrant fibrotic proliferation beneath the pterygium epithelium. This correlates with the expression of epithelial-to-mesenchymal transition (EMT) markers [50]. Our group confirmed the high expression of EMT markers (α-SMA, SNAIL and VIMENTIN) in human primary pterygium cells [34]. Continuous exposure of nicotine and cotinine significantly downregulates the expression of α-SMA and SNAIL. As EMT is related to cell proliferation and migration [51], our findings implied that the retarded cell proliferation and migration of the pterygium cells by nicotine and cotinine could be related to the reduced expression of EMT markers. EMT is a potential mechanism for the effect of nicotine and cotinine on human primary pterygium.

28.2.3 Implication of Nicotine and Its Metabolites on Human Primary Pterygium Cell Development and Progression

Primary pterygium has been linked to the wound healing process [52]. The retarded proliferation and migration properties of human primary pterygium cells by nicotine and cotinine could indicate that the wound healing process by pterygium cells could be reduced upon nicotine and cotinine exposure. Similar findings could be observed in other systems and eye disease that cigarette smoking, nicotine and cotinine hinders the wound healing potential of human periodontal ligament-derived stem cells [53, 54] and RPE cells [55]. Therefore, cigarette smoking as well as nicotine and cotinine exposure could have a generalized effect on wound healing delay in pterygium development and progression. Further investigations are needed to delineate the contribution and mechanism of wound healing process and cigarette smoking in pterygium development and progression.

28.3 Conclusive Remarks

Six hallmark features have been proposed to help to understand the neoplastic diseases [56]. Concerted efforts, including our recent studies, have identified some of these hallmark features in pterygium: sustaining proliferative signaling by expressing the ERK pathway, evading growth suppressors by inhibiting p53 transcriptional activities, and resisting cell death by suppressing apoptosis. We also reported the potential roles

of EMT and ECM remodeling in pterygium, which contribute to another hallmark feature of activating invasion and metastasis. Regarding the feature of replicative immortality, telomerase activities have been reported in pterygium [57]. Angiogenesis-related proteins such as vascular endothelial growth factors have also been reported to be highly expressed in pterygium [58, 59]. These findings allow us to evaluate pterygium in the oncologic perspective. In the future, emerging novel hallmarks including reprogramming of energy metabolism and evading immune destruction in pterygium could be the research directions to better understand this multifaceted disease in the future.

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The Need for Alternative Therapies in Eye Disorders

29

Saurabh Kumar, Shweta Modgil, Kaushal Sharma, and Akshay Anand

Abstract

Eye disorders constitute several vision associated complications that may result in blindness, if not treated on time. Vision researchers have been trying to develop effective treatment strategies but without much success. The continuous failure or ineffectiveness of ocular drugs has led to a desire for alternative therapies. Various preclinical studies have shown that herbal extracts or stem cells can be used as alternative strategies. However, its human benefits can only be accepted after clinical trials. This review provides a new perspective on integrated therapies for eye disorders based on sound scientific evidences compiled from review of the literature. The review provides compelling arguments for much-needed paradigm shift in the face of the failure of current ocular drugs.

Keywords

Ocular · Herbs · Stem cells · Alternative *Prakriti*

29.1 Introduction

Amongst the various disorders, eye disorders are the most devastating ones. The occurrence of eye disease depends on three factors: geographical region, availability, or access to the facilities, and the socio-economic status [1]. Wide range of eye-related diseases such as glaucoma, macular degeneration, retinitis pigmentosa, uveitis, conjunctivitis, retinopathy, etc. [2] demands effective drug targets for prevention of disease progression resulting in blindness. There are only a handful of useful drugs available in the market. Further, these drugs do not precisely act by eliminating the cause of disease, yet the ophthalmic pharmaceutical market is continuously growing at almost 2.5 times the growth rate of the pharmaceutical industry. These are accompanied by serious adverse effects limiting their use.

Previous studies suggest that, as a country becomes wealthier, there is an increase in per capita income, and corresponding increase in such diseases decrease. In poor African countries, the major cause of blindness is cataract and corneal scar. In the middle-income countries like Latin America and India, a majority suffer from

S. Kumar · S. Modgil · A. Anand (✉)
Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India

K. Sharma
Neuroscience Research Lab, Department of Neurology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India

Centre for Systems Biology and Bioinformatics, Panjab University, Chandigarh, India

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glaucoma and diabetic retinopathy. In developed countries, glaucoma and cataract are prevalent [3]. Lack of awareness also contributes to the progression of eye disease [4]. Generally, the ocular drugs are classified into four groups: (1) Lubricants, (2) anti-inflammatory, (3) anti-glaucoma, and antibiotic [5]. Increasingly, these drugs have become resistant to two different ocular bacterial strains: *Streptococcus pneumonia* and *Staphylococcus aureus* [6]. The repeated failure of modern drugs has led the scientific community or ophthalmologists to explore the evidence-based alternative strategies in the form of, herbal remedy, homeopathy [6], yogic techniques and stem cells [7].

29.2 Current Treatment

Eye complications may result in inflammation, redness, itching (allergy), or pain in the eye, which may result in blindness. Various corticosteroids, mast cell stabilizers, or non-steroidal anti-inflammatory drugs (NSAIDs) are used to manage various eye disorders. Depending on the complications and severity of the eye disease, the drugs such as *Lotemax* (loteprednol etabonate), *Durezol* (difluprednate) and *Nevanac* (nepafenac), *Lastacaft* (alcaftadine), *Patanol* (olopatadine), *Optivar* (azelastine hydrochloride), are used either in combination or alone [8]. Artificial tears (*Restasis*) and lubricants are used for dry eyes.

Major success in Ophthalmology was seen in the form of Anti-VEGF therapy for the Age-Related Macular Degeneration (AMD). Ever since the serendipitous discovery of anti-VEGF therapy, the advancement in drug discovery and developments in Ophthalmology are facing challenges. Although the number of compounds being screened for their therapeutic potential has shown a 62% increase, this poses a financial burden for R&D. As a result, the last few decades have seen a decline in the number of FDA (Food and Drug Administration, US) approved drugs. Glaucoma is one of the most common ocular disease treated with either eye drops (prostaglandin, β-blocker, or carbonic anhydrase inhibitors), or surgical procedures. After the first approved medication, latanoprost, two more drugs *Vyzulta* and *Rhopressa* have been added into its approval list [9, 10]. AMD is the disease of old age (generally ≥50), and depending on the condition, it is classified as wet and dry AMD. As of now, there is no available drug for dry AMD), but wet AMD can be treated by *Lucentis* (ranibizumab) and *Eylea* (aflibercept). Although *Avastin* (Bevacizumab) is not approved by the FDA, because of its lower cost, it is more popular amongst the clinicians [11]. Geographic atrophy (GA), another form of macular degeneration, has no effective therapy available till date. Similarly, another prevalent eye disorder with unmet treatment needs is uveitis. It is a disease of intraocular inflammation with no non-corticosteroid drug yet available for its treatment. Retinitis pigmentosa (RP) is another genetic eye disease caused by mutation in *RPE65*. It was in 2017 the first gene therapy, *Luxturna*, was approved by the FDA. The drug is surgically injected into the patient's eye where it delivers a non-mutated copy of the *RPE65* gene which enters the retinal cells and gets replicated in the nucleus [12]. Diabetic retinopathy (DR)/diabetic macular edema (DME), occurring in patients suffering due to Diabetes, is another serious complication that may result in blindness. Chronic DR or advanced DR is characterized by abnormal blood vessels while DME, results from the rupturing of retinal barriers and accumulation of fluid in the macula region. Available approved therapies include anti-VEGF injections, laser treatments, and steroidal implants in the form of *Ozurdex* (dexamethasone) and *Iluvien* (fluocinolone acetonide). Intravitreal corticosteroid implants *Retisert* and *Ozudex* have been approved for posterior uveitis [8, 13]. In 2018, the FDA also approved an artificial intelligence-based device called IDx-DR to detect DR [14] yet therapeutic options remain limited.

29.3 Pitfalls of Current Treatment Modalities

Currently, available treatment strategies either have side effects or are ineffective. The traditionally used eye drops become inefficient because

of the presence of natural barriers and inherent physiology. There are various ways of administration of eye drops. These include systemic (posterior segment), topical, and local ocular (intravitreal, intracameral, retrobulbar, and subconjunctival) [15]. These eye drops have lower ocular bioavailability; as a result these drugs are delivered through invasive technique, implants, or intravitreal injections [8].

The major disadvantage of eye drops is the limited duration of contact between eye drops and their ocular surface. Once these drops are delivered on the ocular surface, it instantly gets diluted by the tear film, and the extra volume spills out. These drops also promote tear production and film rejuvenation, due to which the topical drops get wiped away within 15–30 s by new tear. Besides, the continuous administration of steroids may lead to blindness. The surgical procedures available are painful and sometimes inflammation, increased intraocular pressure (IOP), and hemorrhage may occur in the operated area. A review published by *Santaella* and *Fraunfelder* in *2007* also described ocular toxicity produced by systemic medications [16]. Systemic side effects are higher in children than in adults, because of physiological development. During the administration of eye drops, it is possible that sometimes excess amount (as such drops cannot be weight adjusted) may get absorbed in an unwanted manner.

Intraocular injection of anti-VEGF drugs, however, requires repeated administration and frequent visit to the ophthalmologists for follow up. Also, anti-VEGF therapy is not suitable for all the patients and it is still a mystery why more than half of patients respond poorly without any improvement. In the same way, topical agents recommended for Glaucoma have side effects. However, both new drugs *Vyzulta* as well as *Rhopressa* have presented common side effects such as redness or small bleeds in conjunctiva, eyelash growth, eye irritation/pain [17, 18]. *Luxturna* for RP reported mild adverse events in 73% of patients involving inflammation, nausea, and vomiting highlighting the need for new therapies. Some patients have much serious side effects such as elevated IOP which may result in Glaucoma. The adverse effect of the drugs often results in poor compliance or discontinuation of treatment by patients. In fact, the adverse events are listed as the third common reason for noncompliance to treatment regime [19]. A prospective study on effects of these eye drops on corneal, conjunctival, or palpebral ocular symptoms have shown that 93% patients were experiencing one or the other side effect such as burning sensation, dry eye, blurry vision or corneal tear making patient irritated, dissatisfied, and report low adherence to medication [20].

Antibiotics used for bacterial infections in the eye may cause local intolerance, for example, contact allergic reactions in the case of neomycin. Corticosteroids, when taken in excess, increases the IOP, which may lead to cataract, and systemic toxicity. Vasoactive substances (e.g., Phenylephrine) used to treat allergic reactions are reported to have cardiovascular problems. Children are more prone to the side effects caused by some of the ophthalmic drugs because of the incapability to properly metabolize a drug (CYP deficits) or undeveloped barriers [15]. Maintaining the sterility of eye drops is important and this is achieved by adding preservatives (e.g.; benzalkonium chloride). These preservatives generally do not cause any side effects in case of short-term use. However, the adverse side effects are noted for long-term usage. Chronic conditions like Glaucoma and/or dry eye requires administration for long-term during which the patient may experience pain, itching, or burning sensation [21].

29.4 Failed Clinical Trials

With the growth of drug discovery programs, a variety of compounds, as well as the latest treatment strategies such as gene editing, RNA interference, and targeted drug delivery have been exploited for their use in the treatment of blindness. Although a number of drugs have shown improved outcome at preclinical and early clinical phases, yet most of them are not able to meet the stringent regulatory hurdles at mature clinical stages subsequently leading to drug trial failure.

For example, IL 17A was found to be elevated in the experimental model of uveo-retinitis [22] and inhibited its production exhibiting disease prevention activity. Based on these results, a new drug**,** *secukinumab* (AIN457), a human monoclonal antibody against IL-17A, was developed which further showed positive effects in early trials on patients. Encouraged by these results, when Phase III clinical trial was carried out, it disappointingly failed to match the beneficial effect of earlier trials diminishing the hopes of many patients.

Clinical trials for testing drugs for AMD have witnessed serious failures in the last 2 years. Three drugs *E10030* [23], *lampalizumab* [24], and *OHR-102* [25] having been tested failed at the Phase 3 trial. E10030, which is an inhibitor of platelet-derived growth factor (PDGF) was used in combination with Vascular Endothelial Growth Factor (VEGF) inhibitors for wet AMD. Initial preclinical and Phase I trial supported combinational therapy with a positive response in CNV patients [26]. Subsequent Phase II trial supported the findings from the Phase I study. However, upon review, the trial presented some unexplained results leading to its termination without any success [27]. Similarly, *lampalizumab* was unsuccessful in reducing lesion area in geographic atrophy as was evident from Phase I and II trials [24]. Likewise, Phase III trial of *Fovista* (in combination with either Eylea or Avastin) was met with failure as it was unable to show any better response than Lucentis alone. Therefore, an urgent need for evidence-based alternative approach is imperative.

29.5 Evidence-Based Alternative Treatment Options in Eye Diseases

Since the current traditional drug-based approach of treating various eye disorders does not show permanent or effective cure, people around the world keep opting for herbal formulations as an alternative approach. Besides, the failure of current medical therapies, cost of treatment, unavailability of the equipments, and lack of skilled personnel also contribute towards the need for alternative therapies [3]. It has been seen that the majority of the population residing in developing countries relies on herbal medications. According to the World Health Organization (WHO), 75% of the total world's population is using herbal remedies to treat/improve health complications. It is estimated that more than 53,000 plant species are being used as herbal remedies [28]. However, it is of great concern that most of them are vulnerable to extinction due to various human activities. Ayurveda is an ancient system to deal with diseases and its management as mentioned in Indian Vedas and being practiced since 1500 B.C. Approximately 70–80% of the rural Indian population accepts Ayurveda as the primary health care approach [29]. Many people from different communities: scientific, education, politics, and media elaborate Ayurveda as a home-made remedy which impacts the sustainability of this treatment approach [30], and it is often believed that such remedies are superior over the synthetic drugs and are safe to the living organisms in their natural state [31].

Plants have been widely used for medicinal purposes. In Mexico, there are 4500 reported medicinal plants species. China accounts for almost 5000 of such plant species which have been used as traditional tools [32]. The use of plants for medicinal purposes has been well documented as traditional medicine systems in the form of Ayurveda and Unani in the Indian sub-continent, the Tibetan, Chinese, and Korean of other Asia continent, Amazonian (South America), and other local regions of Africa, and also the Native Americans (North America) [33].

29.5.1 Herbal Based Alternative Therapies

Different plants like onion, garlic, turmeric, *Ginkgo biloba*, catechins, bilberry, blueberry, grape seed extract, green tea, and stilbenes are being used by the people around the world because of their antioxidant, anti-inflammatory,

anti-microbial, and neuroprotective properties. These are rich in a variety of compounds like flavonoids, anthocyanins, resveratrol, vitamin A, E, C, selenium, and carotenoids. Such plant supplements are known to exert their direct or indirect effects on the signaling pathways thereby protecting the retina [34] but these need to be further investigated in detail [35]. These plants are of great importance and their role in protecting the eye disease needs to be validated. Most of them are secondary metabolites and are either phenols or oxygen substituted derivatives like Tannis. 90% of the African and 70% of the Indian population is reliant on these traditional approaches. Even the Chinese hospitals which have separate units for traditional medicine use similar herbs of Chinese origin [36].

About 200 years ago, morphine was extracted by Friedrich Wilhelm Serturner from *Papaver somniferum.* This led to the discovery of the first pharmacologically active compound from plant source [37]. Grape seed extract, onion extracts, curcumin, alpha-lipoic acid, garlic extract, and Vitamin E have been tested by different groups for their beneficial effect in eye disease. Quercetin acts as an antioxidant and helps in the prevention of cataracts [38]. Dietary intake of antioxidants is being examined for its efficacy in AMD and cataracts. Pathophysiology of cataract includes disrupted antioxidant mechanism in the lens because of lower levels of glutathione (a potent antioxidant) [39]. Many cataract-based studies have identified lower levels of glutathione and higher levels of hydrogen peroxide in the eye lens [40, 41]. Glutathione acts by hindering the oxidation reaction of sulfhydryl groups [39]. Resveratrol, a phytochemical present largely in grape skin, has antioxidant, anti-inflammatory, and anti-angiogenic activity and known to induce molecular defenses [42] mediated by defense enzymes (SOD-1, catalase, HO-1) [43]. It has been widely studied in the case of AMD. Various culture and animal studies [44] report its antioxidant effects in protecting the retinal pigment epithelium cells (RPE) from damage induced with acrolein or hydrogen peroxide [45].

A clinical trial was carried out to analyze the combined effect of antioxidants (vitamin C, E, β-carotene, and zinc) and reported a 25% reduction in the progression of age-related eye disease [40]. Patients of glaucoma tend to have increased IOP. It is also reported that cannabinoids decrease [46] the IOP by improving uveoscleral outflow [47]. Curcumin is an active component present in turmeric which has been demonstrated to exert its beneficial effect on eye retinal disease including DR, glaucoma, AMD, retinoblastoma, and retinitis pigmentosa. These effects are exerted through NF-κB, AKT mediated pathway [48]. *Ginkgo biloba* extract is rich in certain flavonoids (quercetin, kaempferol, and isorhamnetin). The in vitro research shows the pretreatment efficacy of GBE rescuing the loss of RPE cells in chronic glaucoma rats [49]. Flavonoids' antioxidant activity is well established in in vitro. However, in vivo efficacy needs comprehensive evaluation. It is believed that flavonoid acts by stabilizing the collagen and enhancing microvascular integrity [50]. Despite the positive role played by plant remedies in treating various eye disease, there has not been much progress in the development of prescription formulation because of the dogmatic approach in research translation.

29.5.2 Yoga Based Alternative Therapy for Vision Related Issues

One of the most significant yogic therapy for vision-related problems [like farsightedness (hypermetropia) or the nearsightedness (myopia)] is *neti kriya*. Nowadays, it is the most prevalent therapy among yogic techniques that is further divided into types the one is *jal neti* and the another one is *sutra neti*. In the *hathyogic* Granth named *Gheranda Samhita,* these two types are broadly elaborated with the procedure for performing *neti*, advantages of *neti*, complications, and many more. Both the methods of *neti* are for the purification of the eye as well as to enhance the vision ratio. In the *jal neti* when

the water enters into the nasal cavity, it influences the organs and nerves related to eye vision, the formation of new blood vessels towards the eyes. It has been found that most of the diseases are managed by this *neti* technique. *Neti* technique not only affects the eye-related problems but also forebrain (like nose, sinuses, eyes, headache problems). In *Gheranda Samhita,* Maharshi Gheranda said that by practicing *neti* one can prevent vision problems.

29.5.3 Stem Cell Therapy as Alternative Approach and Its Complications

Another approach gaining popularity to treat eye disorders involves the use of stem cells which is believed to differentiate into any cell type upon appropriate stimulation. However, this treatment modality is still in its infancy with many challenges with respect to scientific and ethical considerations. The major limitations of using stem cells for treatment include administration to the region of interest and integration into the existing tissue, improperly targeted differentiation, and possibility of tumorigenesis [51]. With the availability of embryonic stem cell lines, stem cell treatment has revolutionized the treatment regimen. However, the safety of these allotransplants is of major concern. In this context, the American Academy of Ophthalmology released a statement in 2016 stating that there are no FDA approved stem cell therapies for any eye-related disorders emphasizing that the safety of these is yet unknown [52]. A recent study reported severe adverse effects of autologous adipose tissue-derived stem cells injected intravitreally into the eye. Visual acuity in these patients dropped down to 20/200 from the baseline range of 20/30 to 20/20 in 1 year. Vision loss was associated with other complications such as lens dislocation, ocular hypertension, hemorrhagic retinopathy, vitreous hemorrhage, or combined retinal detachment [53]. Despite many limitations, preclinical studies and associated clinical trials involving stem cells are increasingly being tested for ocular disorders.

29.6 Ambiguous Genetic Analysis: Limitation of Current Reductionist Approach

Most of the retinal diseases are polygenic hence making them cumbersome to treat. Despite abundant knowledge of genetic components for retinal diseases, there is a lack of concrete treatment strategies in the field. Most of the treatments are based on data derived from the use of reductionist approach, i.e., targeting of a single genetic component to combat the disease. However, the success rate with such an approach is limited and confined to the symptomatic relief [54].

We have already discussed the failure of various clinical trials and the limitation of cell-based therapies in the field. However, complete genetic screening of an individual can provide the risk factors of a given patient. Sometimes, the clinical manifestations could be ambiguous but analysis of genetic components can accurately influence clinical outcomes. Additionally, the screening of genetically susceptible risk factors can lead to good clinical categorization beneficial for personalized treatment. Such data could be useful for better management for such complex retinal diseases [55].

The therapeutic strategies based on single gene approach do not consider the importance of non-coding mutations that fall in the intronic regions of the gene. Similarly, the impact of copy number variants (CNVs) in long interspersed genetic regions and haplotype variations could also be considered to make the pragmatic solution for such diseases. Moreover, the influence of rare variants and variants of uncertain significance (VUSs), by applying various bioinformatic and network approaches, including GeneMANIA, could also provide the better analysis to discern the complexities in retinal disease [54–56]. Functional evaluation of such genetic variations can be done in vivo or in animal cells which may provide the biological significance and could also be related to clinical investigations of an individual. Additionally, most of gene-based therapies have shown limited promise due to non-consideration of genetic modifiers (environmental factors). Therefore, the varied degree of penetrance may differ in clini-

cal and therapeutic outcomes. Ethnicity, gender, geographic distribution, microflora, flora and fauna can contribute equally towards the clinical tranalational of a disease. The development of personalized medicine acknowledges the role of genetic contribution in the treatment modalities that requires further investigation for better clinical outcomes [57].

29.6.1 Ayurgenomics and Development of Personalized Medicine

Ayurveda argues that an individual has own internal constituents (called *Prakriti*) which provides him/her internal susceptibility combat against diseases and response to the environment, and also varies with *Prakriti* of the individual. In the modern era, we described susceptibility against both environment and disease, depending up the genetic constituents of an individual (SNPs or copy number variations). Integrative approach by merging both Genetics and Ayurveda (called Ayurgenomics) could assist in developing a striking and translational approach in order to deal with prevailing complex disease phenotypes [58, 59]. The *prakriti* of an individual could be derived based on the proportion of all three entities (*tridoshas*), including *vata, pitta, and kapha*. All *tri-doshas* are governed by both genetic components as well as the environment factors like the mother diet and daily lifestyle. The *prakriti* can also be influenced by birth pace, ethnicity, and family characteristics of an individual and will remain unchanged throughout the life. Systemic analysis based on ayurgenomics approach can be stratified the individual genetics in which expression level and genetic variations (leading to differential response for disease) could be regulated through *prakriti* of an individual [58, 59].

It is desirable to also consider such coding, non-coding, and copy number changes to deal with such heterogenic and complex retinal diseases which may provide the raw material for the development of personalized or precision medicine. After the introduction of the GWA study concept in 2005, it was demonstrated that the most common coding genetic variant Y402H was associated with 43% AMD cases [60]. However, subsequent GWAS has defined various genetic loci that have been found most frequent in AMD patients and may vary based on the clinical manifestation of AMD, i.e., dry, wet, and geographic atrophy. However, allelic and non-allelic interactions could also alter the genetic and clinical outcomes of the individual. Hence, the treatment strategies must include such investigations to deal with such biological complexities and disease manifestations. Interestingly, responses to two different antioxidants like vitamins and zinc supplementations could also alter based on the risk alleles of CFH and ARMS2. Studies signifying the pharmacogenomic categorizations of AMD cases could facilitate the development of precision medicine [61]. Similarly, the anti-VEGF responses for AMD pathology were also found to be associated with the Y402H SNP variation of an individual and could confer the protective factor for disease [62–65]. These studies suggest the need for pharmacogenomic predictor and precise clinical translation for patients suffering from retinal disease.

The holistic approach is taking into consideration the correlation of the genetic outcome and *prakriti* types of an individual. Various holistic approaches including *tratak* practice and analysis of *prakriti* of an individual can also pave the way to providing the precise treatment regimen for complex diseases like retinitis and AMD. It has been recently demonstrated that the *EGLN1* protein was differentially expressed based on the *prakriti* types of the individual. Moreover, TT genotype (rs479200) of *EGLN1* was also differentially distributed between individuals and was more frequent in *kapha prakriti* and positively correlated with the expression of *EGLN1*gene in comparison to *pitta prakriti*. Such investigation was done on high altitude population; mostly suffer from pulmonary edema [65]. Therefore, the integrative approach to correlate pharmacogenomic (treatment response) with ayurgenomics (*prakriti* types) along with consideration of environmental factors and allelic/non-allelic analysis could provide deeper insights in the development of personalized medicine.

29.7 Summary

Modern science lacks complete knowledge for the permanent treatment of different eye diseases including Glaucoma, AMD, RP, etc. The ocular drops or drugs are either ineffective or possess side effects. Ocular surgeries are also costly and painful and at times inaccessible. A dysfunctional eye cannot provide proper vision and if not diagnosed or treated, may result in complete vision loss. Several government policies have been implemented to create awareness to prevent blindness. Therefore, there is an alarming need to develop effective alternative formulations or strategies in the form of herbal remedies, yogic practices, or stem cell therapy. This provides a cost-effective way to deal with ocular problems.

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Gene Therapy for Retinal Diseases

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Chitra Gopinath, Subhradeep Sarkar, and Arkasubhra Ghosh

Abstract

Inherited retinal dystrophies (IRDs) are caused by mutations resulting in progressive functional loss of photoreceptors. The onset of these disorders could be by birth or affect an individual across various ages. Patients diagnosed with Leber congenital amaurosis, retinitis pigmentosa, Stargardt disease, macular dystrophies, choroideremia, etc. experience gradual vision impairment or blindness. Several genes responsible for these dystrophies are known based on extensive genetic studies which led to an understanding of their structure, function, and involvement in cellular pathways making them potential targets for therapeutics. Gene therapy using various delivery vectors such as recombinant adeno-associated virus (rAAV) as a treatment modality offers hope in such conditions that currently have no cure. This chapter provides an overview of different retinal diseases, key genes involved and their mutations resulting in pathological and clinical features, and gene therapy approaches applied. Safety and efficacy are the primary considerations for any gene therapy study. Developments in vector design, promoter modifications, split-gene strategies to express large expression cassettes, compatible vector serotypes or strains to use for efficient retinal cell transduction, alternate gene delivery systems, immune challenges such as the presence of neutralizing antibodies and other toxicity would be given special emphasis in this chapter. Some of the recent success stories of retinal gene therapy preclinical studies and clinical trials are discussed.

Keywords

Inherited retinal dystrophies · rAAV · Gene therapy · AAV serotypes · Retina · Animal models

30.1 Introduction

Over the last several years, the knowledge of genetics and genetic mutations driving various diseases including inherited retinal diseases has grown exponentially. This cumulative knowledge from human and animal model studies raised the hope of genetic therapies (Fig. 30.1). The eye is an easily accessible and fascinating organ for gene therapy. Gene delivery is safer as the eye is highly compartmentalized and has immune privilege to a certain extent. The presence of blood–retina and blood–aqueous barriers limit infection processes or immune activity and thus also hin-

C. Gopinath · S. Sarkar · A. Ghosh (✉)
Grow Research Laboratory, Narayana Nethralaya Foundation, Bangalore, India
e-mail: arkasubhra@narayananethralaya.com

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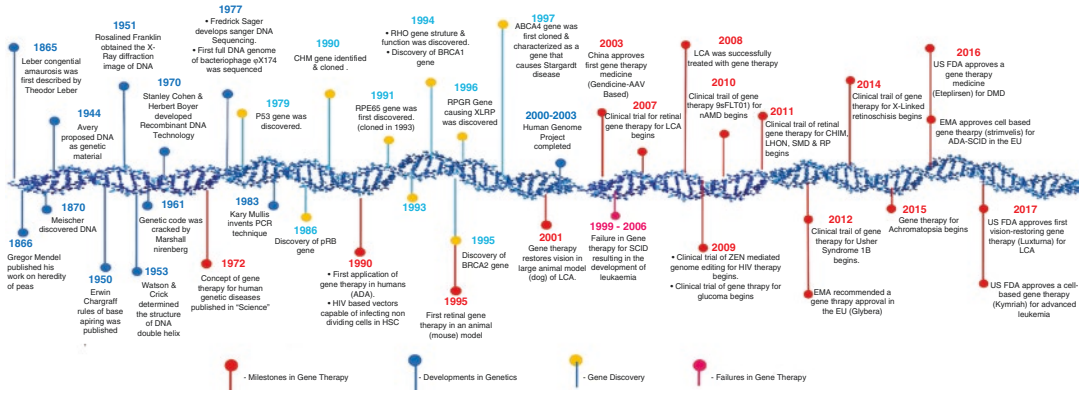

Fig. 30.1 Milestones in retinal gene therapy. The progress in understanding diseases with discoveries in genetics and remarkable technological developments in the field of molecular biology and applications leading to gene therapy products are time lined in this figure

ders gene delivery parenterally to the typical ocular targets such as the retinal pigment epithelium (RPE) and neuronal retina. Thus, gene delivery to the internal layers of the eye depends on ingenuous surgical methods developed over the past few decades such as subretinal injection, supracameral injections, intravitreal injections, etc. Such methods deliver the genes directly to the target tissues while minimizing immune responses outside of the eye. Lack of active intraocular immune responses in the eye also protects vector transduced cells from being lost due to rejection. Most cellular layers in the eye do not replicate and hence a single appropriate dose of viral vector carrying the therapeutic gene is required for efficient transduction and prolonged gene expression. Though recombinant adeno-associated virus (rAAV) has a packaging capacity of around 4.7 kb, it is the vector of choice in clinical trials for ocular gene therapy. Intravitreal and subretinal injections of AAV serotype 1, 2, 5, 7, and 8 have been successfully used for high-level, long-term gene expression in retinal cells [1, 2]. Large genes (example: *ABCA4* and *USH2A*) can be accommodated with improved vector design strategies such as overlapping, trans-splicing, and dual-hybrid vector systems while using rAAV [3]. Other gene delivery methods such as integration deficient lentivirus (IDLVs) to overcome risks of insertional mutagenesis and nanoparticle-based (such as liposomes, polymers, and peptide compacted DNA) have also been studied and successfully used in vivo on retinal cell types. Different nanoparticles have different biochemical properties that govern their internalization, endosomal escape, and transportation to the nucleus. Efficacy of gene transfer can be observed by simple noninvasive procedures like electroretinography and fundus examinations. Availability of both small and large animal models that mimic human disease conditions for several monogenic inherited retinal dystrophies (IRDs) makes it convenient to test strategies for therapeutic benefits in preclinical studies.

Retinal degeneration can be grouped under three broad classifications—(1) hereditary or inherited retinal dystrophies, (2) retinal degenerations, and (3) retinal dystrophies that are part of a syndrome. Some of the most common monogenic IRDs include several forms of retinitis pigmentosa (RP), Leber congenital amaurosis (LCA), Stargardt disease (STGD), choroideremia, achromatopsia, X-linked juvenile retinoschisis (XLRS), Usher syndrome, and other cone–rod and rod–cone dystrophies (CRDs and RCDs). Age-related macular degeneration (AMD), diabetic retinopathy (DR), vein occlusions, and retinopathy of prematurity (ROP) belong to a multifactorial class of degenerations. Similar retinal conditions could be part of syndromes such as Usher, Bardet–Biedlt, and others. Different genes encoding enzymes or structural components contributing to the visual cycle or retinal structure are responsible for various types

of retinal disorders. The retina is a highly specialized structure consisting of light-sensitive cone and rod photoreceptor cells, which initiate neuronal signaling in response to light stimulation. The photoreceptor cells are supported by a monolayer of polarized retinal pigmented epithelium cells (RPE), which performs many key processes including the regeneration of visual pigment that is bleached following light exposure (the visual cycle). Key genes such as retinal pigment epithelium 65 (*RPE65*) retinoid isomerohydrolase, ATP binding cassette subfamily A member 4 (*ABCA4*), MER proto-oncogene tyrosine kinase (*MERTK*), nuclear hormone receptor (*NR2E3*), etc. are responsible for the proper functioning of retinal cells facilitating essential processes such as phototransduction and homeostasis required for normal visual function. The RPE cells are located between the neural retina and the choroid and plays a critical role in the maintenance of visual function. RPE receives light, phagocytoses photoreceptor outer segments, participates in circadian rhythm, is involved in fatty acid metabolism, forms the outer blood–ocular barrier which maintains the subretinal space, performs repair and renewal of cells [4, 5]. RPE cells are polarized and maintain tight cell junctions. Disruption of RPE phagocytosis has been linked to disease phenotypes such as STGD disease and RP [6]. Therefore, the selection of patients for gene therapy requires prior knowledge of the mutated gene as well as the mode of inheritance. Sections in this chapter would include further details on all important considerations for a safer and highly efficacious gene transfer and recent developments in gene therapy, both preclinical and clinical trials for retinal dystrophies.

30.2 Recombinant Adeno-Associated Virus Vectors in Retinal Gene Therapy

Recombinant AAV used in gene therapy contains two inverted terminal repeats (ITRs) retaining the cis genome packaging signal. The *rep* (replication) and *cap* (capsid formation) genes required for virus production are supplied on a trans-helper plasmid. The DNA and promoter of interest placed in between the AAV ITRs is the transgene expression cassette [7, 8]. It can efficiently transduce nondiving cells and is nonpathogenic, which makes it safe for use in gene therapy. There are different strategies to expand the packaging capacity of AAV beyond the traditional 4.7 kb. One such approach is to make a truncated version of a large gene by excluding certain sequences without compromising protein function and retaining functionally relevant sequences [9]. Other strategies such as the cis-activation approach involve dividing the expression cassette into two parts (dual vectors)—one containing the promoter plus enhancer and the other containing the gene are packaged individually. These would get reconstituted upon co-transduction and concatemerization of the ITRs [10]. Additional dual vector approaches include trans-splicing, overlapping, and hybrid strategies. In the trans-splicing approach, the expression cassette is spilt into promoter plus 5′ half of coding sequence and splice donor signals in one vector and the other containing a splice acceptor signal with the 3′ half of the coding sequence. Reconstitution would occur when the 5′ and 3′ vectors form head-to-tail concatamers. Expression is achieved when the intervening double-ITR structure is removed from the mature mRNA using the host cell splicing machinery [11]. Overlapping dual vectors use recombinogenic sequences located in the middle of the gene where the two parts of the gene share an overlap sequence. Upon co-infection with the vectors carrying the two parts, the intact full-length gene is reconstituted by homologous recombination initiated at the overlap to generate the full-length expression cassette, such as alkaline phosphatase (AP) [3]. However, the trans-splicing vector efficiency is dependent on the splice site whereas the overlapping vector efficiency depends on the recombinogenic potential of the overlap sequence. The hybrid dual vector strategy is independent of the transgene properties as it is a novel combination of trans-splicing and overlapping systems [12]. The dual vector strategies may still not meet the need for gene therapy of larger genes (>8.5 kb) such as *CDH23*

known to cause Usher syndrome type ID. A triple AAV system to expand the cargo limits to 14 kb has been tested and shown to be 40% successful compared to single vector systems [13]. In general, the dose of virus, transgene properties, site of injection and serotype used are factors that contribute to an effective gene therapy without side effects of cellular toxicity [14]. A brief compilation of all the preclinical studies (Table 30.1) and AAV based clinical trials (Table 30.2) thus far are provided.

30.2.1 Serotypes for Retinal Cell Transduction

Most IRDs originate from the retinal pigment epithelium cells (RPE) or rod and cone photoreceptors. To choose an AAV serotype that is specific and best for transduction of retinal cell types via any route of administration has been critical. Animal retina, which differ structurally as well (depending on species) to the human retina do not always recapitulate the same surface receptors as human retinal cell types for specific tropism. Ex vivo human retina and retinal pigment epithelium-choroid explants were used to check for transduction efficiency and tropism of AAV2/1, AAV2/2, AAV2/4, AAV2/5, AAV2/6, AAV2/8, and AAV2/9 carrying green fluorescent protein (GFP) driven by cytomegalovirus (CMV) promoter. AAV2/4 and AAV2/5 efficiently transduced photoreceptor cells, the latter being highly specific to the outer nuclear layer (ONL). AAV2/8 exhibited comparatively lower transduction of photoreceptors, whereas higher levels of transduction were observed in the inner retina. AAV2/8 also showed a preference to cone cells in particular. Good transduction of retina is achievable as seen in the pig model injected with AAV2/8 subretinally, suggesting some degree of difference in tropism across species [15]. Interestingly, retinoschisis and CHM gene therapy studies have used the AA8 serotype for gene delivery.

Greater understanding of AAV biology helped design strategies which could overcome proteasome degradation of vectors by incorporating point mutation of surface-exposed tyrosine to phenylalanine (Y-F) in the capsid of rAAV 2, 8, and 9 and achieve greater levels of transduction of retinal cells compared to their wild-type counterparts [16–18]. In a retinal degeneration study, three of the most efficacious AAV capsids AAV2/8(Y733F), AAV2/2(quad Y272, 444, 500, 730F) and AAV2/(7m8) were tested for transduction efficiency in an *rd1* mouse model, monkey and human retinal explants. AAV2/2(7m8) resulted in a greater area of retinal transduction and the highest percentage of gene expression. Transduction of cell types and efficacy of AAV2/2(7m8) and AAV2/2(quad Y-F) via intravitreal or subretinal routes of delivery were similar and better compared to AAV2/8(Y733F). AAV2/2(7m8) was extremely efficient in transducing all retinal cell types compared to the other serotypes which selectively transduced few cell types [19]. rAAV2 (triple Y − F + T − V) efficiently transduced photoreceptors by intravitreal injections [20]. rAAV2/9 and 2/8 transduce RPE, photoreceptors (PR), Muller cells (MC), inner nuclear layer (INL), outer plexiform layer (OPL), and ganglion cells (GC) of mouse and dog retinal cells efficiently and result in high transgene expression [21, 22]. rAAV2/8 and rAAV2/7 are capable of infecting rods and cones at high levels of transduction efficiencies compared to AAV2/5 [23]. Subretinal delivery of rAAV2/5 and rAAV-2/4 carrying CMV. GFP were injected to dog, mouse, and macaque. rAAV2/5 transduced rods and cones better than RPE cells, whereas, with rAAV2/4, transduction was restricted to RPE cells and resulted in long-term gene expression [24]. There has been no report of successful transduction of retinal cells using rAAV2/3 serotype. RPE cells to some extent do get transduced by rAAV2/6 serotype [25]. rAAV/rh10 has been shown to efficiently transduce mice photoreceptor cells and rescue rhodopsin deficient phenotype [26]. rAAVShH10 (close variant of AAV6) has a greater tropism for Muller glial cells as tested in a rat model of RP [27, 28]. A summary of serotypes and their tropism is represented in Table 30.3.

Table 30.1 Preclinical AAV mediated gene therapy studies

AAV mediated retinal gene therapy (Preclinical studies)						
Sl. No.	Human gene	Disorder	Delivery method	Gene therapy for target species	Gene therapy strategy	AAV serotype
1	PDE6B	Autosomal recessive retinitis pigmentosa	Subretinal	Rd1; Rd10 mice	Augmentation; single-stranded oligonucleotide-mediated gene repair	AAV5, AAV8
2	4 Sulfatase	Mucopolysaccharidosis VI	Subretinal	MPS VI Siamese cat	Augmentation	AAV2
3	TYR	Oculocutaneous albinism	Subretinal	Tyrosinase; albino 2 Jackson mice, GPR 143+ mice	Augmentation	AAV
4	ABCA4	Autosomal recessive retinitis pigmentosa, Stargardt 1,cone–rod dystrophy	Subretinal	Abcr^{+} mice	Augmentation	AAV5
5	MYO7A	Usher syndrome 1B (retinitis pigmentosa)	Subretinal	Shaker1 mice	Augmentation	AAV5
6	AIPL1	Retinitis pigmentosa, cone dystrophy, Leber's congenital amaurosis	Subretinal	AIPL1 Hypomorph mouse	Augmentation	AAV8, AAV5, AAV2
7	MERTK	Leber's congenital amaurosis, retinitis pigmentosa	Subretinal	RCS rat	Augmentation	Ad; AAV
8	BBS-4	Bardet-Beidl retinitis pigmentosa	Subretinal	Bbs-4^{+} mice	Augmentation	AAV
9	LRAT	Leber's congenital amaurosis, retinitis pigmentosa	Subretinal	Lrat^{+} mice	Augmentation	AAV
10	CHM	Choroideremia	Subretinal	NAP	Augmentation	AAV2
11	L-Opsin	Red-Green color Blindness (XL)	Subretinal	Squirrel monkey	Augmentation	AAV5
12	CNGA3	Achromatopsia, cone dystrophy	Subretinal	Cnga3^{+} mice	Augmentation	AAV8
13	IMPDH1	Autosomal dominant retinitis pigmentosa 10	Subretinal	Impdh1^{+} mice	Augmentation	AAV
14	CNGB3	Achromatopsia, cone–rod dystrophy	Subretinal	Cngb3^{+}	Augmentation	AAV8
15	CNAT2	Achromatopsia	Subretinal	Gnat2 (Cpfl3) mice	Augmentation	AAV5
16	GUCY2D	Leber's congenital amaurosis	Subretinal	GC1^{+} mice; retinal degeneration chicks	Augmentation	AAV8
17	Whirlin	Usher Syndrome 2D (retinitis pigmentosa)	Subretinal	Whirlin^{+} mouse	Augmentation	AAV2/AAV5
18	RHO	Autosomal dominant retinitis pigmentosa	Subretinal	Rho^{+}, pro23H mouse	Augmentation, zinc finger-based transcriptional repression	AAV
19	RPE65	Leber's congenital amaurosis, retinitis pigmentosa	Subretinal	Rpe65^{+} mice and dog	Augmentation	AAV

Some of the gene therapy studies for candidate genes which are carried out in small and large animal models of various retinal diseases are compiled in this table

Table 30.2 AAV mediated retinal gene therapy clinical trials

AAV mediated retinal gene therapy (Clinical trials)							
Sl. No.	Disease	Human gene	AAV serotype	Delivery method	Trial phase	Sponsor	References
1	Leber's Congenital Amaurosis (LCA 2)	RPE65	AAV2	Subretinal	Phase 3 completed	Spark Therapeutics	A
		RPE65	AAV2	Subretinal	Phase 2 completed	U. College London	B
		RPE65	AAV2	Subretinal	Phase 1 ongoing	U. Pennsylvania, NEI	C
		RPE65	AAV2	Subretinal	Phase 2 ongoing	AGTC	D
2	X-Linked Retinoschisis	RS 1	AAV 2tYF	Intravitreal	Phase 2 ongoing	AGTC	E
		RS 1	AAV 8	Intravitreal	Phase 2 ongoing	NEI	F
3	Choroideremia	CHM	AAV2	Subretinal	Phase 2 completed	U. Oxford (NightstaRx)	G
		CHM	AAV2	Subretinal	Phase 2 ongoing	U. Alberta (NightstaRx)	H
		CHM	AAV2	Subretinal	Phase 2 ongoing	Spark Therapeutics	I
		CHM	AAV2	Subretinal	Phase 2 ongoing	Bascom Palmer (NightstaRx)	J
	Choroideremia	CHM	AAV2	Subretinal	Phase 2 enrolling	U. Tubingen	K
4	LHON	ND4	AAV2	Intravitreal	Phase 2 completed	GenSight Biologics	L
		ND4	AAV2	Intravitreal	Phase 1 ongoing	NEI, Bascom Palmer	M
		ND4	AAV2	Intravitreal	Phase 3 ongoing	GenSight Biologics	N
5	Achromatopsia B3	CNGB3	AAV 2tYF	Subretinal	Phase 2 ongoing	AGTC	O
6	Achromatopsia A3	CNGA3	AAV8	Subretinal	Phase 2 ongoing	U. Tubingen, LMU Munich	P
		CNGA3	AAV 2tYF	Subretinal	Phase 1 enrolling	AGTC	Q
7	Retinitis Pigmentosa	MERTK	AAV	Subretinal	Phase 1 ongoing	King Khaled Eye Specialist Hospital	R
		PDE6B	AAV	Subretinal	Phase 2 ongoing	Horama	S

Reference	Link
A	https://clinicaltrials.gov/ct2/show/NCT00999609
B	https://clinicaltrials.gov/ct2/show/NCT00643747
C	https://clinicaltrials.gov/ct2/show/NCT00481546
D	https://clinicaltrials.gov/ct2/show/NCT00749957
E	https://clinicaltrials.gov/ct2/show/NCT02416622
F	https://clinicaltrials.gov/ct2/show/NCT02317887
G	https://clinicaltrials.gov/ct2/show/NCT01461213
H	https://clinicaltrials.gov/ct2/show/NCT02077361
I	https://clinicaltrials.gov/ct2/show/NCT02341807
J	https://clinicaltrials.gov/ct2/show/NCT02553135
K	https://clinicaltrials.gov/ct2/show/NCT02407678

Table 30.2 (continued)

Reference	Link
L	https://clinicaltrials.gov/ct2/show/NCT02064569
M	https://clinicaltrials.gov/ct2/show/NCT02161380
N	https://clinicaltrials.gov/ct2/show/NCT02652780
O	https://clinicaltrials.gov/ct2/show/NCT02599922
P	https://clinicaltrials.gov/ct2/show/NCT02610582
Q	https://clinicaltrials.gov/ct2/show/NCT02935517
R	https://clinicaltrials.gov/ct2/show/NCT01482195
S	https://clinicaltrials.gov/ct2/show/NCT03328130

Various clinical trials for candidate genes are presented in this table. The sub-table lists the references for the respective trials

Table 30.3 Retinal cell tropism of rAAV serotypes

AAV serotypes	Animal models	Human retinal explants and clinical
rAAV 2/1	RPE	RPE, PR
rAAV 2/2	RPE, PR	RPE, PR
rAAV 2/3	–	–
rAAV 2/4	RPE	RPE, PR
rAAV 2/5	RPE, PR	RPE, PR, ONL
rAAV 5/5	RPE, PR	No data
rAAV 2/6	RPE	RPE
rAAV 2/7	RPE, PR	RPE, PR
rAAV 2/8	RPE, PR, INI, GC	RPE, PR
rAAV 2/9	RPE, PR, INL, GC	RPE, PR
rAAV2/8 (Y733F)	RPE, PR	RPE, PR
rAAV2/2 (quad Y-F)	RPE, PR	RPE, PR
rAAV2/2 (7m8)	All retinal cell types	All retinal cell types
rAAV2/2 (triple Y–F+T–V)	PR	PR
rAAV4/4	RPE	No data
rAAV/rh10	PR	No data

Different serotypes of AAV exhibit selective transduction of the retinal cells, showing varied tropism across species. *RPE:* retinal pigmented epithelium, *PR:* photoreceptors, *INL:* inner nuclear layer, *ONL:* outer nuclear layer, *GC:* ganglion cells
Source: GROW Lab

30.2.2 Broadly Active Versus Specific Promoters

To ensure cell type-specific gene expression, use of a cell-specific or gene-specific promoter is essential to circumvent unwanted transgene expression at off-target areas. An efficient promoter driving high and clinically relevant levels of therapeutic gene expression is necessary so that a single appropriate dose of the vector would be sufficient for treatment. This would overcome consequences of immune response or cellular toxicity resulting from multiple or high virus dosage. Gene therapy studies over the past decades have used broadly active promoters such as CMV [29], human ubiquitin C promoter (UbiC) [30], and chicken beta-actin promoter (CAG) [31]. Some retina-specific promoters used are the RPE-specific promoter—Best1 (bestrophin-1) [32] and RPE65 promoter [33], photoreceptor-specific promoters such as human red opsin (RedO) [34, 35], human rhodopsin (Rho) [23, 35], human rhodopsin kinase (RK) [36], mouse cone arrestin (CAR) [37], etc. Choosing a promoter needs careful assessment as some promoters could pose a certain level of toxicity to the retina such as shortening of the cone outer segment, reduction of the outer nuclear layer, and dysmorphic pigment epithelium [38]. Toxicity due to AAV has been observed in some studies involving animals and humans. Sheep with achromatopsia were treated with AAV2-CNGA3 at a high dose of 10^{12}, showed retinal atrophy and lymphocytic infiltration [39]. Another study, involving NHP eye treated with subretinal AAV8-CNGA3 showed responses of both innate and adaptive immunity [40]. An LCA2 gene therapy clinical trial reported strong evidence of an inflammatory response in five out of eight individuals treated with the higher dose of AAV2-RPE65 [41]. The Alberta choroideremia gene therapy clinical trial, reported

an adverse effect resulting in functional loss of the subject's retina [42]. Broad expression promoters typically may have higher expression levels compared to tissue-specific ones. An example is a study which compared transgene expression by five different promoters—cytomegalovirus immediate-early gene promoter (CMV), human desmin (Des), human alpha-myosin heavy chain (α-MHC), rat myosin light chain 2 (MLC-2), and human cardiac troponin C (cTnC) to drive *LacZ* mediated by AAV9 intravascular delivery in mice. CMV outperformed other tissue-specific promoters resulting in the highest level of transgene expression [43].

30.2.3 Injection Methods

Intravenous injection of AAV would not deliver the therapeutic gene to the target site due to the presence of the blood–retina barrier. Therefore, AAV must be delivered directly to a specific site in the eye where the RPE and photoreceptors are transduced easily (Fig. 30.2). The space between the RPE and photoreceptors, the subretinal space, is the most effective and preferred site for gene therapy. High titer virus preparations are typically required for ocular administration so that the injection volume of the vector may be minimized. In this procedure, a parafoveal bleb is created by subretinal injection of normal saline followed by injection of the vectors through the same self-sealing retinotomy. Preoperative interventions include optical coherence tomography (OCT), pars plana vitrectomy, inner limiting membrane (ILM) staining. Postoperative follow-ups can be done using OCT and fundus scans [44]. During the subretinal injection procedure, there is a risk of reflux of vector into the vitreous which may lead to vitritis due to immune reactions to the capsid.

On the other hand, intravitreal injection is less invasive but may be disadvantageous due to the dilution of the virus within the vitreous or presence of neutralizing antibodies (nAbs) leading to poor transduction of retinal layers [45]. ILM is a wall separating the vitreous and neural retina. A recent injection method called the "subILM," a surgical route to the space between the ILM and neural retina could better serve the purpose of overcoming issues of intravitreal injections [46]. On the basis of AAV being thermostable and negatively charged, yet another recent development was to enhance AAV uptake by applying an in vivo low electric current across the eye to further enhance retinal transduction after intravitreal administration. The electric current was reported to be safe and does not cause damage to the retina structure or function as tested in a wild-type mouse retina using AAV8 [47].

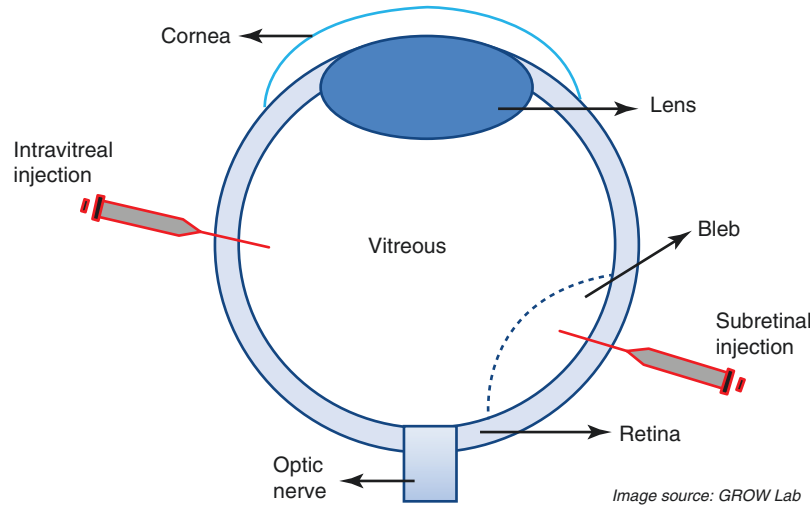

Fig. 30.2 Injection sites for AAV administration. The intravitreal and subretinal spaces are two common sites of injection to deliver therapeutic AAV particles. In the subretinal route, a bleb is created separating the RPE layer and photoreceptors. The intravitreal route is a less invasive procedure where the viral particles are delivered in the vitreous humor

30.2.4 Immune Responses, Biodistribution, and Cellular Toxicity

Recombinant AAVs are known to be nonpathogenic and less toxic. Humans or other animals are naturally exposed to AAV and this poses a challenge to the safety and efficacy of therapy using wild-type AAV capsid. Approximately 80% of the population show the presence of nAbs (neutralizing antibody) to wild-type AAV2 capsid [48–50]. Retina has an immune privilege due to the existence of the blood–retina barriers; therefore, vector administration via the subretinal route has a much reduced risk of negative immune response due to previous exposure to AAV. This is supported by the observation that subretinal readministration of virus led to repeated successful transduction even in the presence of nAbs to AAV in the serum [51, 52].

Post subretinal delivery of AAV2/8 vectors in a canine model, systemic distribution of AAV may occur in other parts such as the brain probably due to trans-synaptic transport to the neurons. This may not necessarily lead to further effects of the transgene if expressed under retinal cell-specific promoters as reported by Stieger et al. [53]. Several years post successful subretinal transfer of AAV 2, 4, and 5, in dogs and primates, existing AAV particles were observed in the outer plexiform layer (OPL) and in all other layers of the retina [54]. Dosage of virus in most of the in vivo gene therapy studies use a range of 10^8–10^{13} vg copies/ml in a single dose. Since the subretinal space can accommodate merely a few microliters, hence a higher viral tier is dosed thereby raising a risk for local tissue reactions, but the currently used injection methods have been shown to be relatively safe with the edema resolving uneventfully in most cases. Other general cellular toxicities arising due to the nature of transgene and transcriptional elements have to be assessed for morphology, physiology and inflammation status in preclinical models before proceeding to clinical trials [38].

30.3 Alternate Methods of Gene Delivery

Apart from AAV, a variety of other vectors have been used to treat retinal diseases which are compiled in Table 30.4. In 1997, lentiviral vectors (LV) carrying GFP driven by CMV or rhodopsin promoter were subretinally injected to the retina of newborn and adult rats and expression followed over 12 weeks. CMV promoter resulted in GFP expression in both photoreceptors and RPE, whereas rhodopsin promoter-driven expression was restricted to the photoreceptors. Due to lack of interphotoreceptor space, expression in adult rat retina was observed only at the site of injection, unlike newborn animals. This long-term expression of the transgenes in photoreceptors could be due to stable integration of the transgene into the genome of the host cell as demonstrated before [55]. The transduction efficiency of LV was similar to adenovirus (Ad)-based vectors [56, 57]. AAV performed better in transducing retina compared to LVs and Ads [58, 59]. Integrase deficient lentiviral vectors (IDLVs) have shown sustained gene expression in vitro and in vivo [60]. These episomal IDLVs are suitable for delivering large genes and transducing nondiving cells in the retina and neural retina without risks of insertional mutagenesis. In 2006, successful use of second-generation self-inactivating (SIN)-IDLVs delivered subretinally in adult rodent models of retinal degeneration ($Rpe65_{rd12/rd12}$ mouse and *Mertk*-deficient rat) showed long-term (9 and 3 months' follow-up in mice and rats, respectively) eGFP expression in adult RPE cells. Thus, IDLVs are potential candidate vectors for gene therapy of retinal dystrophies.

Nanoparticles (NPs) such as polymers, liposomes, peptide compacted DNA have been tested as gene delivery systems for retinal diseases [61, 62]. The advantage of using NPs is the ease in manipulating its chemical properties to suite DNA delivery, low cost of manufacturing, and transferring large vectors without any immune reactions. Critical steps in gene transfer

Table 30.4 Non-AAV mediated retinal gene therapy studies

NON-AAV mediated retinal gene therapy						
Sl. no.	Disease	Gene	Vector	Delivery method	Pre-clinical	Clinical
1	Stargardt disease	ABCA4	Equine Infectious Anemia Virus (EIAV)	Subretinal	$Abcr^{+}$ mice	Phase 2 ongoing https://clinicaltrials.gov/ct2/show/NCT01367444
2	Usher Syndrome 1B	MYO7A	Equine Infectious Anemia Virus (EIAV)	Subretinal	Shaker1 mice	Phase 2 ongoing https://clinicaltrials.gov/ct2/show/NCT01505062
3	Leber's congenital amaurosis	GUCY2D	Lenti virus	Subretinal	$GC1^{+}$ Mice; retinal degeneration chicks	–
4	Leber's congenital amaurosis, retinitis pigmentosa	RPE65	Lenti virus	Subretinal	$Rpe65^{+}$ mice and dog	–
5	Autosomal recessive retinitis pigmentosa	Channel rhodopsin-2	Electroporation	Subretinal	Rd1, Rd10, rd16 mice	–
6	Light damage	Catalase	Adeno virus	Subretinal	Light-damaged mice	–
7	CNV	Retinostat	Equine Infectious Anemia Virus (EIAV)	Subretinal	Laser photocoagulation in mice	Phase 2 ongoing
8	Macular dystrophy	PeripherinRDS	Compacted DNA nanoparticles	Subretinal	Rds^{+}, R172W tg Mouse	–

Lentivirus, equine infectious anemia virus, adenovirus, and nanoparticles are some of the alternate gene delivery methods to treat genetic retinal diseases, some of which are currently in preclinical and clinical trial phases

via NPs involve uptake by target cells, escape endosomal degradation in the cytosol, and transport of genetic cargo to the nucleus without causing cytotoxicity and should be biodegradable in the human body [63]. NPs are taken up by RPE cells by either endocytosis [62]. NPs enter photoreceptors and glial cells by different methods of endocytosis depending on their shape, size, charges, and amount of DNA load they carry [64]. Measures to aid critical processes for efficient gene transfer and expression are considered for prospective human applications. Numerous types, modifications, or customizations of NPs are being studied to make them efficient carriers. The PLGA (poly lactic-co-glycolic acid) and PEG (polyethylene glycol) compacted DNA NPs are the forerunners for safe delivery of genes to photoreceptor cells and the RPE [65]. Cationic liposomes were first used for gene transfer to rat eye via subretinal or intravitreal injections resulting in expression of the lacZ reporter transgene in ganglion cells and RPE alone with no expression observed in photoreceptor cells. This could be due to the phagocytic process of RPE actively taking in most of the NPs compared to less efficient endocytosis by rods and cones [66]. A detailed review of various NPs for ocular gene therapy can be found in Adijanto and Naash's article [64].

30.4 Gene Therapy of Congenital Retinal Degenerations

IRDs are inherited in the family in either autosomal dominant, autosomal recessive, or X-linked manner. The presence of mutational heterogeneity in autosomal dominant conditions is a challenge for gene therapy due to the toxic "gain of function" of the mutant allele. Such conditions lead to the death of photoreceptor cells [67]. The approach to treat a dominant-negative condition typically involves silencing of the dominant allele that is detrimental for the cell, followed by replacement with a codon optimized version of the gene resistant to the silencing. Ribozymes or small interfering RNA (siRNA) are used for silencing the defect. Some common inherited retinal conditions, preclinical studies, and recent clinical trials will be discussed in the following subsections.

30.4.1 Retinitis Pigmentosa (Rod–Cone Dystrophies)

Retinitis pigmentosa (RP) is a progressive rod–cone degeneration (RCDs) caused by mutations in more than 200 genes identified thus far, which affects 1 in 4000 individuals. RP is inherited as autosomal dominant (30–40%) (for example *RHO* gene; 25% of adRP), autosomal recessive (50–60%) (for example, *USH2A* gene; 20% of arRP), X-linked conditions (5–15%) (for example, *RPGR* gene; 70% of XLRP) or some rare forms such as mitochondrial diseases [68, 69]. Mutations in genes responsible for loss of photoreceptors leads to early signs such as difficulty in dark adaptation and night blindness as the rods get affected first. This is followed by a gradual decrease in the visual field (tunnel vision) progressing to complete loss of vision. Electroretinogram (ERG) usually shows a decline in photoreceptor activity in patients with RP. Phenotype and age of onset are highly variable with individuals.

More than 150 mutations in the rhodopsin (*RHO*) gene belong to the G-protein coupled receptor family and present on chromosomal location 3q22.1, which leads to adRP. The structure and function of rhodopsin was described in 1994 [70] The rhodopsin protein is bound to 11-cis retinal (vitamin A) which gets activated upon light stimulation. This event initiates a chain of chemical reactions to produce an electric signal which is sent to the brain and perceived as vision. Gene therapies for *RHO* adRP were either focused on minimizing the expression of the toxic mutant allele or designing a mutation-independent strategy. In a recent gene therapy study, the authors developed a highly efficient shRNA that is specific to human and canine *RHO* in a mutation-independent manner. This vector design also involved the human RHO cDNA, codon-optimized to make it resistant to RNA interference, with both the shRNA and the

RHO gene being expressed from a single AAV virion. This vector was tested in a spontaneously occurring dog model of *RHO*-adRP. The native canine RHO RNA was completely inactivated via subretinal vector delivery leading to expression up to 30% of normal cellular expression. OCT imaging and histopathology of the treated area showed normal structure and presence of normal RHO protein in the remaining transduced photoreceptors. Long-term follow-up of greater than 8 months by OCT and ERG showed reversal of phenotype and a stable maintenance of photoreceptor structure and function. This successful animal model study can be applied to treat patients with this form of adRP by gene therapy [71].

Rare XLRPs are caused by mutations in *RP2* and *OFD1* gene. *RPGR* gene present on chromosomal position: Xp11.4 was identified to cause XLRP in 1996 [72]. This encodes for retinitis pigmentosa GTPase regulator that is necessary for cells' ciliary function aiding vision and is the most common form of XLRP. Along with few naturally occurring [73] and genetically modified mouse models [74], canine models with *RPGR* mutations such as the Siberian husky reported in 1999, which a spontaneous model of XLRP that mimics the human disease [75]. A recently reported model with a deletion of exons 1–4 in *RPGR* gene is the Weimaraner dog, a naturally occurring model for XLRP [76]. *RPGR* gene is a purine-rich gene which is prone to genetic instability, making it difficult to manipulate [77]. In a 2012 preclinical study, rAAV2/5 vector carrying *RPGR* cDNA driven either by a human photoreceptor-specific IRBP (interstitial retinol-binding protein) or GRK1 (rhodopsin kinase) promoter and was delivered at a dosage of 10^{13} vector genome/ml (vg/ml) via the subretinal route. This was not successful as the mice developed toxicity to the mutated therapeutic cDNA [78, 79]. A different report described an RPGR vector strategy where the purine-rich region was deleted in frame resulting in long (deletion of 314 codons) and short (deletion of 126 codons) forms in AAV8.GRK1.RPGR^{ORF15} vectors. The long form showed functional restoration of the photoreceptors in the *Rpgr*-null mouse, whereas the short version did not fare well. This could be due to the maximal glutamylated status of the protein which is required for full therapeutic activity of the protein in the full-length RPGR which may have decreased in proteins formed from reduced sequences [80]. These challenges were overcome by using a codon-optimized version of the gene which is highly stable. AAV. coRPGR^{ORF15} was used to treat *Rpgr*-null and *Rd9* mice mouse models which showed reversal of the phenotype without any toxic side effects, thereby establishing the first successful proof-of-concept leading to three clinical trials initiated in 2017 and 2018 [81]. First clinical trial by Nightstar Therapeutics using AAV8.GRK1.RPGR^{ORF15} (NCT03116113), second by MeiraGTx UK Ltd. using AAV2/5.hRKp.RPGR (NCT03252847), and the third by Applied Genetic Technologies Corp (AGTC) using AAV2/2(YF).GRK1.RPGR^{ORF15} (NCT03316560) [82].

An example of autosomal recessive RP caused by mutations in the *MERTK* gene resulted in the accumulation of outer segment debris due to defective RPE phagocytosis, which is necessary for the renewal of photoreceptor outer segment [83]. In a preclinical study, rats were treated with subretinal injection of AAV-*MERTK* vectors that demonstrated significant improvements in response to ERG [84]. Six patients with confirmed mutations in MERTK were treated by gene therapy in a phase 1 clinical trial. Three out of the six patients had shown improvements in vision and had no signs of systemic toxicity in long-term follow-ups of over 2 years [85]. A 2019 gene therapy phase 2 clinical trial reported final outcomes of six male patients across different age groups affected with choroideremia who had received subfoveal injection of AAV2-REP1 at 10^{11} vg/0.1 mL. These subjects who had documented a reduction in the vision now demonstrated improvements in visual acuity without any adverse toxic effects [86].

30.4.2 Cone–Rod Dystrophies

Gene therapy for retinitis pigmentosa GTPase regulator interacting protein 1 (*RPGRIP1*)- in a canine model of severe cone–rod dystrophy

(CRD) was performed using AAV5 and AAV8. Cone and rod functions were restored and *RPGRIP1* was stably expressed over a period of 2 years in all treated eyes. This large animal model of CRD provides hope toward the treatment of patients [87]. Achromatopsia is caused by mutations in cyclic nucleotide-gated channel alpha 3 (*CNGA3*) (other genes causing similar phenotype are *CNGB3*, *GNAT2*, *PDE6C*, *PDE6H,* and *ATF6*) (http://www.sph.uth.tmc.edu/RetNet/) resulting in poor visual acuity, photophobia, and inability to recognize colors due to cone dysfunctions. The presence of a naturally occurring *CNGB3*-sheep model facilitated the study of treatment strategies [88]. In a 2011 study, sheep deficient in *CNGA3* were treated unilaterally with AAV5 vectors carrying either the mouse or the human *CNGA3* driven by a cone-specific 2.1-Kb red/green opsin promoter [89]. Follow-up studies after 6 years showed the animals had normal vision restored and were measured as demonstrated by ERG and other functional tests. This led to the initiation of clinical trials in *CNGA3* achromatopsia patients (NCT02935517 and NCT02610582) [39].

30.4.3 LCA

Leber Congenital Amaurosis (LCA), first described by Theodore Leber in 1869, encompasses a set of autosomal recessive congenital rod–cone dystrophies (RCDs). The prevalence ranges from 2 to 3 in every 100,000 newborns and is the major cause of blindness in children. Around 15 genes (*IMPDH1, AIPL1, CRB1, CEP290, CRX, GUCY2D, LRAT, RD3, RDH12, MERTK, RPGRIP1, TULP1, SPATA5, RPE65,* and *LCA5*) (http://www.sph.uth.tmc.edu/RetNet/) that are required for normal vision are involved whose mutation result in LCA early in childhood. These genes are responsible for the development of photoreceptor cells, phototransduction and phagocytic processes in normal retina. Other structures such as cilia are needed for the perception of vision. Mutations in *CEP290* (15%), *AIPL1*(12%), *GUCY2D* (12%), and *RPE65* (8%) genes are the most common with other gene mutations accounting for a small percentage (5%) of LCA patients.

Gene therapy for RPE65 in a naturally occurring canine model of RPE65 with visual impairments similar to that observed in human LCA type II were treated with subretinal injection of rAAV-RPE65. The dogs demonstrated improvements in that visual function establishing a proof-of-concept to treat LCA [90]. A long-term follow-up of over 3 years showed stable expression of RPE65 in target areas of the treated eyes and recovered significant retinal function as demonstrated by ERG and other tests [91]. In 2007, the first clinical trial (later two additional trials followed) for LCA had begun with reports of the initial phase of clinical trials in 2008 stating that AAV-RPE65 used were safe and effective over a follow-up of 1 year post treatment in patients [92, 93]. Thereafter, successful Phase I to III clinical trials of gene therapy of RPE65 using Luxturna were reported leading to its approval by the FDA for commercial use in 2017 [94, 95].

30.4.4 Stargardt Disease

Stargardt disease (STGD1) is a juvenile macular degeneration with a prevalence of 1 in 8000–10,000 individuals [96]. However, the age of onset could be during adolescence or any time in adulthood [96]. During the visual cycle, all-trans-retinal is transported out of the discs into the cytosol by ATP binding cassette subfamily A member 4 (ABCA4) transmembrane transporter in the photoreceptors where it gets converted to all-trans-retinol by dehydrogenases (RDH8). Phototransduction results in the formation of bisretinoid A2E (fluorophore of lipofuscin), a by-product that is toxic. The ABCA4 protein is responsible for the removal of these substances out of the cell. A nonfunctional ABCA4 results in the accumulation of these toxic substances causing the death of photoreceptor and atrophy of surrounding RPE cells [97]. Patients with mutations in the *ABCA4* gene experience progressive central vision loss leading to blindness due to death of the photoreceptor cells [98, 99]. Currently, there is no cure for ABCA4 mutation related

to Stargardt disease. ABCA4 cDNA is large (6.8 kb) which would require a suitable vector system such as dual AAVs, IDLVs, or compacted DNA nanoparticles for efficient gene delivery and expression (refer Sect. 30.2 of this chapter). *ABCA4* null mice exhibit phenotypic expression similar to humans [100]. Nanoparticles have been studied in STGD1 mice models where CK30PEG carrying human ABCA4 cDNA and human interphotoreceptor retinoid-binding protein (IRBP) or mouse opsin (MOP) promoters were tested. Expression of ABCA4 was observed at 2 and 8 months post injection and had rescued the phenotype [101]. Traditional AAV vectors are not efficient in the packaging and transduction of large genes [102]. Ghosh lab in India [3] and MacLaren lab in the UK are involved in strategies to package *ABCA4* as split gene into dual AAV vector systems toward treatment for STGD1. Recently, therapeutic levels of *ABCA4* were achieved using the overlapping AAV strategy and which showed the first proof-of-concept in *ABCA4* knockout murine model. Truncated proteins that are formed due to dual vector strategies were reduced by optimizing recombination. Functional ABCA4 protein was observed in photoreceptor outer segments of the mice retina with a successful reversal of the phenotype [103]. This approach could thus be applied to a large animal model followed by clinical trials for gene therapy of Stargardt disease using dual AAVs.

30.4.5 X-Linked Juvenile Retinoschisis

X-linked juvenile retinoschisis (XLRS) is early-onset macular degeneration occurring in males with a prevalence of 1:5000 to 25,000 males worldwide. This condition results from mutations in (*RS1*) gene encoding retinoschisin 1 protein required for cell adhesion, organization, and structural maintenance of the retina. Patients experience poor vision, accompanied by congenital nystagmus, strabismus, vitreous hemorrhage, retinal detachment leading to blindness in severe forms [104]. Preclinical gene therapy studies for retinoschisis were carried out in rabbits and the RS1 knockout mice, where intravitreal administration with self-complementary AAV8-scRS/IRBPhRS showed rescue of the disease phenotype [105, 106]. These successful results led to the initiation of clinical trials and recent reports of phase I/IIa are reported by Cukras et al., [107].

30.5 Gene Therapy of Retinal Neovascularization

Certain retinal pathological conditions like diabetic retinopathy (DR) [108] and age-related macular degeneration (AMD) [109] are caused due to hypertrophic, neovascular formations in the retina and choroid. These are relatively common conditions leading to age-related progressive blindness. AMD affects individuals of age 50 and above. Currently, FDA-approved treatment for these conditions are repeated intravitreal injections of antibodies against VEGF (vascular endothelial growth factor). AAV mediated gene therapy for DR involves strategies to protect nerves and blood vessels from damage or by inhibiting the neovascular networks and vascular hyperpermeability. Antibody approaches targeting sFlt-1, Flt23k, and PEDF have been studied on small and large animal models [110–114]. Other targets to inhibit angiogenesis that are being tested are endostatin, angiostatin, and metalloproteinase-3 [115–117]. Gene therapy to prevent neovascular formations in the case of wet AMD involves AAV2 vectors carrying sFLT-1 and sFLT01 that have been studied in animal models as well as phase 1 and 2 clinical trials. Reports of these studies have demonstrated the treatment approach to be safe and effective in correcting the vision of the majority of patients in clinical trials [118–122].

30.6 Gene Therapy of Syndrome-Associated Retinal Degenerations

Usher syndrome type I is caused by mutations in the myosin VIIa gene (*MYO7A*), present in the RPE. Patients with this syndrome

experience early-onset RP phenotype and hearing loss by birth. A murine model, the shaker1 (*Myo7a*$^{sh1\text{-}4626SB}$) mouse, has been extensively used for preclinical studies [123]. In 2007, the first gene therapy study using third-generation self-inactivating LVs encoding a CMV promoter-driven *MYO7a* gene were administered subretinally in the eyes of the shaker1 mice. The reversal of the phenotype was observed as the ciliary function was restored [124]. A recent study used subretinal injection of an equine infectious anemia virus (EIAV) vector system carrying the *MYO7A* gene driven by CMV promoter, which led to the production of the protein and restoration of vision in knockout mice. Safety was assessed in monkeys which consequently led to the development of UshStat, for clinical trials of Usher type 1B syndrome gene therapy [125].

30.7 Conclusion

The genetic basis of a disease and phenotypic variance were discovered and described by scientists and clinicians from the 1800s (refer Fig. 30.1) There has always been hope to cure or correct the mutations that lead to hereditary disorders. Over the past two decades, gene therapy has developed from ideation to proof-of-concept to clinical trials being conducted across the world. Of all diseases, gene therapy for monogenic inherited diseases, retinal diseases in particular have reached clinical trials early and have now been approved for treatment in many countries. Viral and nonviral methods have been explored for their gene delivery efficiencies to treat various dystrophies. Strategies to overcome and assess adverse immune reactions, toxicities, insertional mutagenesis without compromising gene delivery and expression efficacies are key to a successful gene therapy. Proof-of-concept in vitro and in vivo animal model studies are the initial supportive data that are essential for a treatment approach to achieve clinical approval. With successful clinical reports of patients treated with gene augmentation therapies for retinal dystrophies, "future looks brighter" indeed for other ocular disorders.

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31 The Use of Human Pluripotent Stem Cells (hPSCs) and CRISPR-Mediated Gene Editing in Retinal Diseases

Grace E. Lidgerwood, Alex W. Hewitt, Alice Pébay, and Damián Hernández

Abstract

The human retina is one of the most complex tissues of the body, composed of various specialized cells organized in a fashion that enables the reception, conversion, preliminary-processing and final transmission of light signals to the brain. Dysfunction of any of the retinal cell types essential for normal vision ultimately leads to vision decline and potentially blindness. Diseases affecting the retina and optic nerve can broadly be divided into two forms, either complex or monogenic diseases. Complex, multifactorial diseases include age-related macular degeneration and glaucoma. Rarer heritable retinopathies and optic neuropathies, often affecting the young, include Stargardt Disease, Usher Syndrome, Leber's Hereditary Optic Neuropathy, Best Disease, Choroideremia and Retinitis Pigmentosa. To understand the genetic and pathological features of retinal diseases, it is imperative that appropriate models and technologies are implemented to enhance the prospects of therapies for patients. For the purpose of this chapter, we will focus on the current technologies used for generating retinal disease models *in vitro*, and how gene-editing technologies such as CRISPR/Cas are propelling ophthalmic research into the spotlight.

Keywords

Human pluripotent stem cells · Gene editing CRISPR · Disease modelling · Retinal differentiation

Alice Pébay and Damián Hernández contributed equally.

G. E. Lidgerwood
Department of Anatomy and Neuroscience, The University of Melbourne, Parkville, Australia

Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, East Melbourne, Australia
e-mail: grace.lidgerwood@unimelb.edu.au

A. W. Hewitt
Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, East Melbourne, Australia

School of Medicine, Menzies Institute for Medical Research, University of Tasmania, Hobart, Australia

A. Pébay (✉)
Department of Anatomy and Neuroscience, The University of Melbourne, Parkville, Australia

Department of Surgery, Royal Melbourne Hospital, The University of Melbourne, Melbourne, Australia
e-mail: apebay@unimelb.edu.au

D. Hernández
Department of Anatomy and Neuroscience, The University of Melbourne, Parkville, Australia
e-mail: damian.hernandez@unimelb.edu.au

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31.1 Current Models for Eye Disease

Currently, few models exist that accurately capture the complexity of human retinal diseases. The preferred animal models of retinopathies and optic neuropathies include primates, given their close genetic similarity to humans and shared anatomical features; however, they are expensive and require comprehensive housing and considerations, making them beyond the reach of many research laboratories [1]. Rodents are commonly used to investigate eye diseases; however, despite being a powerful model organism, they have some notable limitations. Around 1% of human genes have no identifiable mouse homologues [2] and anatomically they differ greatly from humans: the eyes of rats and mice do not have maculae or foveae and 85–90% of their optic nerve axons decussate to the other side of the brain [1]. Consequently, mouse models do not always fully replicate the features of human retinal diseases. Primary cultures from animals and human cadavers are a popular in vitro method of studying ocular diseases; however, the limitation of using primary tissue is the time from death to culture, which often cannot be controlled, resulting in tissue degradation [3]. Immortalized cell lines overcome this issue; however, a number of established lines fail to exhibit morphological characteristics of the native tissue [4].

31.1.1 Human Pluripotent Stem Cells

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), provide a logical solution to address concerns around the aforementioned limitations of animal and primary tissue models. Pluripotent stem cells (PSCs) theoretically can give rise to any somatic cell type in the adult, and indeed protocols to differentiate stem cells into many functional somatic cell types have been established *in vitro*. The first hESC line was derived by Thomson and colleagues, who in 1998, successfully isolated the inner cell mass of a preimplantation embryo and demonstrated that it was capable of self-renewal and germ layer differentiation [5]. Human adult somatic cells can also be reprogrammed back into a pluripotent-like state (induced pluripotent stem cells or iPSCs) using various methods to induce expression of particular transcription factors. In 2006, Takahashi et al. discovered that the expression of four transcription factors—octamer-binding transcription factor (*OCT3/4* or *POU5F1*), sex-determining region Y (*SOX2*), Kruppel-like factor 4 (*KLF4*), and myc-proto-oncogene (*MYC*) *[6]*—was sufficient to induce reprogramming into iPSCs [6, 7]. The addition of *NANOG* and *LIN28* (replacing *KLF4* and *MYC*) was also shown to be sufficient to induce conversion of somatic cells to iPSCs [8]. These first methods of reprogramming began using integrative retroviral [7] and lentiviral [8] systems; however, various modifications have since been made (with different combinations of transcription factors) in an attempt to increase transfection efficiency and reduce risks associated with integrative approaches. To date, several non-integrative approaches have been established, including adenoviral [9], Sendai virus [10–12], protein [13, 14], mRNA [15], miRNA [16–18], vector [19–21] or episomal plasmids [22].

The ability to generate patient-specific iPSCs brings the possibility to manipulate or correct genes associated with particular diseases, opening the prospect of gene therapy for monogenic and multigenic diseases. This will be discussed in more details later in this chapter, when the use of CRISPR in iPSCs and disease models are introduced. Drug discovery and screening is particularly pertinent to iPSCs, creating the possibility of personalized therapies for patients. Finally, it offers the prospect of autologous cell replacement therapy, minimizing the risk of immune rejection in the patient receiving the treatment [23]. Current human clinical trials of hPSC-replacement therapies largely centre around hESC-derived RPE for the treatment of age-related macular degeneration (AMD), Stargardt disease and Retinitis pigmentosa; however, progress is being made with iPSC-derived cells and other retinal cell types that are currently being validated in animal models (reviewed in [24]).

31.2 Retinal Development

Given the strength of hPSC-derived models lies in part in their ability to mimic developmental processes that occur *in utero*, it is worth introducing the developmental steps that lead to the formation of the human retina. In mammals, the retina is a developmental appendage of the neural ectoderm [25]. During the final stage of neural tube development, two invaginating structures emerge, known as the optic vesicles [25]. In this phase of development, retinal progenitors positive for Ceh-10 Homeodomain-Containing Homologue (*CHX10)*, Melanogenesis-Associated Transcription Factor (*MITF)* and Visual System Homeobox 2 (V*SX2)* are found [26]. The distal part of the tube forms contacts with what will become the lens, and *MITF* expression is downregulated, not surprising given this is the precursor for premelanosome protein (*PMEL)* expression, a gene that confers pigmentation to the retina [26]. These $MITF^{-ve}$ cells will mature to eventually give rise to the neural retina progenitors, whilst the $MITF^{+ve}$ cells will eventually give rise to the outermost layer of the human retina, the retinal pigment epithelium (RPE) [26].

The apical neural retina undergoes a second invagination, positioning itself adjacent to the newly forming, $MITF^{+ve}$ RPE layer. This stage heralds the formation of the optic cup. Progenitors of the optic cup divide symmetrically before halting and undergoing asymmetric division, seeding daughter cells that will eventually adopt distinct fates [27]. This process is tightly regulated, under the control of both intrinsic and extrinsic factors. As the optic cup neural retina progenitors mature, the earliest retinal neurons appear, including ganglion cells, horizontal cells amacrine cells and cone photoreceptors emerge [28]. The last retinal neurons to emerge are the rod photoreceptors, bipolar cells and the retinal glia known as Müller cells [28]. Further photoreceptor differentiation occurs even later, where specialized rod photoreceptors form, and the colour-detecting cones branch off into specialized sub-cone populations (red, blue and green-light wave-detecting subtypes). This stage is closely monitored by specific expression of various photoreceptor-specific transcription factors including Neural Retina Leucine Zipper (NRL), Cone–Rod Homeobox (CRX) and Orthodenticle Homeobox 2 (OTX2) [29–31].

The optic cup formation and maturation can be mimicked in vitro by stimulating the signalling pathways involved in this process in cultures of human pluripotent stem cells (hPSCs). To date, a range of protocols have been developed to differentiate hPSCs to various retinal cell types, as discussed below.

31.2.1 Differentiation of PSCs into RPE Cells

The RPE is a monolayer of polygonal, pigmented cells that plays an essential role in maintaining the health and function of photoreceptors, and functionality of underlying vasculature, known as the choroid. Among its many critical functions, the RPE forms the outer blood–retinal barrier (BRB), creating a physical barrier that separates the immune-privileged retina from circulating factors. The RPE supplies and transports nutrients and waste products to both the photoreceptors and choroid; it phagocytoses and recycles photoreceptor outer segments (POS)—a waste product of visual cycling—and protects the retina against photo-oxidation by effectively absorbing light [32]. Dysfunction of the RPE can lead to photoreceptor dystrophy and blindness, such as that associated with AMD and other genetic diseases including Best disease, Retinitis Pigmentosa, Scoresby and Stargardt disease.

A variety of protocols have been designed to differentiate hPSCs to RPE cells, each involving varying degrees of complexity and efficiency. hPSCs can spontaneously differentiate to RPE cells when basic Fibroblast Growth Factor (bFGF) is removed; however, this method is labour-intensive and time-consuming, requiring manual excision of pigmented foci, followed by their dissociation and expansion [33–36]. Efficiency is improved when a guided and sequential differentiation protocol is implemented. Using various combinations of growth factors at different time points, hPSCs can con-

sistently and efficiently differentiate to form cultures rich in RPE cells. RPE can be propagated using either suspension, embryoid body formation; or adherent monolayer cultures in combination with a range of growth factors cocktails. The bone morphogenetic protein (BMP) and Activin/Nodal signalling pathways play important roles in the development and specification of RPE fate—initial inhibition of BMP and Activin signalling is critical for neural ectoderm specification [37, 38], whilst reactivation of these pathways later enables differentiation to the RPE [39, 40]. Current guided methods for differentiating hPSCs to RPE include combinations of growth factors including bFGF, BMP antagonist Noggin, the Wnt antagonist Dickkopf-1 (DKK1), nicotinamide, Activin A, casein kinase I inhibitor 7, the ALK4 inhibitor SB-431542 and the Rho-associated kinase inhibitor Y-27632 [33, 39, 41–44]. Formation of eyecup structures in a defined medium, prior to RPE enrichment has also been shown to be a feasible approach [42, 45–47].

31.2.2 Differentiation of hPSCs to Neural Retina Using an Adherent Model

A number of adherent neural retina differentiation approaches were established in the early 2000s using mouse embryonic stem cells in a stepwise approach with defined growth factors and/or co-culturing with retinal tissue [48–50]. In 2005, the first demonstration of telencephalic differentiation and subregional specification (including the presumptive optic cup) was reported in mouse ESCs. Using the Serum-free Floating Embryoid Body aggregate (SFEB) approach (a combination of embryoid body and subsequent adherent culture step) their method sat at the intersection of traditional adherent 2D approaches and the advent of the first three-dimensional suspension culture methods [51]. Later that year, Ikeda et al. improved on the existing SFEB method, reporting for the first time that the resulting population contained photoreceptor-positive markers indicative of putative rod and cone cells. In a step further, Osakada et al. showed that human ESCs could also be differentiated into retinal progenitors [52]. Differentiation of human iPSCs to RPE and retinal progenitors was later demonstrated using a suspension culture exposed to Wnt and Nodal antagonists, with a photoreceptor-enrichment step using retinoic acid and taurine [53]. During the era of the SFEB differentiations, Lamba et al. reported a variation of the SFEB method using a combination of embryoid body and adherent cultures in a defined, growth factor-mediated media containing blood serum substitutes N2 and B27, Noggin, DKK-1 and IGF-1 [54]. They also reported a successful method to differentiate human PSCs into retinal cells, albeit more rapidly, however a majority of progenitors gave rise to mostly primarily functional inner retinal neurons (ganglion and amacrine cells) and was notably absent of photoreceptor progenitors. An improvement on this method was reported by Zhou et al. with the addition of the multifunctional BMP/TGFβ/Wnt antagonist COCO [55].

These early retinal differentiation protocols pioneered the field of *in vitro* retinal development; however, the derived cells largely lacked the organization required to accurately model the intricate structure of the retina adequately. Furthermore, no functionality was largely void in the aforementioned studies. Nevertheless, findings from these early works paved the way for the next phase of retinal modelling—three-dimensional retinal organoids.

31.2.3 Three-Dimensional Retinal Organoids to Yield Retinal Progenitors and Mature Retinal Neurons

The formation of the optic cup and subsequent maturation phase is a carefully timed and staged process, involving sequential expression and/or silencing of various eye field genes at critical timepoints. This process can be mimicked in vitro through specific culturing conditions, generally involving the addition or subtraction of specific growth factors involved in the developmental process. The resulting structures form three-dimensional retinal organoids, exhibiting struc-

tures, functions and morphologies that closely resemble the earliest stages of foetal eye development [56]. An advantage of retinal organoids is that they naturally form into structures containing stratified layers of all retinal neurons. Not only does this provide the necessary environment to foster cell–cell interaction and communication that is undoubtedly vital for cell maturation and function, it provides the opportunity to isolate individual cell types of interest to study in isolation; up until today, there are still no methods of making all retinal neurons without using the organoid approach. One of the first optic cup differentiation protocols was published by the Sasai group, who demonstrated that mESCs could develop into retinal organoids in vitro with minimal media consisting of sequential stages of 1.5% KSR and N2 respectively [57]. The inclusion of an extracellular matrix of Matrigel provided a critical scaffold matrix for improved formation of fully formed optic cups. Simultaneously, Meyer and colleagues showed that human iPSCs differentiated to retinal organoids using an adaption of their earlier methods [56]. Both studies highlighted that their respective "minimal media" methods naturally promoted a neuroepithelial cell fate, indicating that minimal intervention led to a system that naturally favoured neuroepithelial cell fate.

In 2012, Nakano described a method to promote neurogenesis and retinal cup formation using a directed, growth factor-mediated methodology in an entirely three-dimensional floating culture system [46]. Other studies then demonstrated that hiPSCs can recapitulate steps of retinal development observed in vivo and form three-dimensional retinal cups that contain all major retinal cell types arranged in their proper layers [58]. Up until this publication, bipolar and Müller cells had been noticeably absent from the organoids. The derived optic cups formed from adherent culture, after being manually excised and maintained in a suspension culture. Importantly, the photoreceptors in this hiPSC-derived retinal tissue achieve advanced maturation, showing the beginning of outer-segment disc formation and photosensitivity [58]. Retinal organoid differentiation efficiency was improved by Reichman et al., bypassing embryoid body formation and eliminating the use of exogenous molecules, coating, or Matrigel [59]. The addition of the Notch inhibitor DAPT was also shown to greatly improved the population of photoreceptor precursors [59]. With time, protocols have become more complex, using combinations of adherent, plated and suspension cultures with various growth factor formulas. This results in increased functional RGC [60, 61] and photoreceptor [62–65] yields. Importantly a number of these have been shown to integrate into the mouse retina, an important step for assessing the therapeutic potential of iPSC-derived retinal tissue [64, 66–68].

31.3 Gene Editing in Retinal Degenerative Diseases

31.3.1 Clustered Regularly Interspaced Short Palindromic Repeats (CRIPSR)/Cas System

The CRISPR/Cas system originated from the type II CRISPR-Cas systems used by bacteria to protect from viral and plasmid intrusion in an adaptive immune response and has now been adapted for mammalian nuclear genome editing [69–71]. The CRISRP/Cas system is composed of the endonuclease CRISPR-associated protein Cas, and a duplex RNA (tracrRNA;crRNA) sequences that binds to the Cas and forms base pairs DNA target sequences. This allows a site-specific double-strand DNA break. Compared with previous gene-editing technologies that require extensive protein engineering (such as zinc finger and transcription activator-like effector nucleases), CRISPR/Cas only requires changes in the crRNA sequence to allow the targeting of any specific DNA sequence of interest. The introduction of DNA breaks by Cas protein activates an intrinsic DNA repair mechanism in mammalian cells. The non-homologous end joining repair can often result in DNA base-pair insertions or deletions (indels) that can disrupt a gene, the homology-direct repair uses a donor homology template to repair by homology recombination, with the

introduction of an exogenous DNA template specific point mutations can be modified. Briefly, this allows the interruption of specific genes or the introduction of specific point mutations of interest. The increasing use of CRISPR/Cas system has broader its application for the generation of cell lines, the establishment of animal models and gene therapy for different genetical diseases including of the retina.

31.3.2 Gene Editing of PSCs with CRISPR/Cas

Inherited retinal dystrophies are characterized by the loss of photoreceptors and/or RPE cells leading to vision loss [72]. Currently, over 250 genes have been identified as causative of inherited retinal dystrophies [73]. Monogenic diseases are an attractive target for gene editing since only one locus needs to be modified. Gene editing technology advances using CRIPSR/Cas combined with pluripotent stem cells (PSCs) has accelerated the generation of reliable models of disease [74]. The ability to repair or introduce genetic mutations on disease or healthy PSC lines and the potential of PSC to differentiate into any cell type, including of the retina, provides a platform for compound testing, disease modelling and preclinical studies.

Retinitis Pigmentosa (RP) is a leading cause of inherited blindness worldwide. More than 85 genes have been identified as causative of RP, yet with mechanisms underlying pathology remaining poorly understood. One of the most prevalent causative genes is the *retinitis pigments GTPase regulator* (*RPGR*) [75]. *RPGR* encodes for a protein with two major isoforms, the default one and the one containing ORF15 coding-exon [76]. The ORF15, which is only expressed in photoreceptors, contains a substrate of glutamylation that seems to be essential for the function of photoreceptors [77]. Several studies using animal models have been used to understand the disease mechanisms of *RPGR* [78–80]; however, differences in the gene sequence between species represents a challenge for the development of an appropriate human model. Interestingly, the generation of iPSCs from RP patients containing different mutations in *RPGR* have been differentiated into RPE cells and retinal organoids that recapitulate the pathogenesis of RPGR mutation such as decreases in retinal gene expression, photoreceptor cell numbers and electrophysiology, and cilia length [81]. Following correction of the *RPGR* mutation in iPSCs using CRISPR/Cas, the derived-photoreceptors recovered the gene expression, electrophysiology and morphology [81]. Other iPSCs have also been generated with different mutations in *RPGR* and corrected isogenic controls have been generated using CRISPR/Cas9 [82].

Leber congenital amaurosis (LCA) is another inherited retinal dystrophy for which iPSC lines have been generated; however, no isogenic lines have been reported so far [83]. A variant of LCA (LCA10) is caused by mutations in *CENTROSOMAL PROTEIN 290* (*CEP290*), that encodes for a centrosomal protein localized in the connecting cilium of photoreceptors [84]. When iPSCs with mutations in *CEP290* were differentiated into optic cups, a decreased *CEP290* expression was noted and a cellular phenotype was observed, with mislocalization of RPGR leading to a disrupted ciliogenesis [85]. However, the control iPSCs used in this study were generated from fibroblasts obtained from individuals without the mutation in *CEP290,* not through a gene correction. A different group has generated isogenic lines from the excision of a splice mutation in *CEP290* with CRISPR/Cas9 and demonstrated correction of the transcript and protein in patient iPSCs [86].

Another study has examined the cellular pathology of splicing factor autosomal dominant retinitis pigmentosa (*RP13*) caused by a mutation in the *PRPF8* gene [87]. iPSCs were generated from a patient with a missense mutation in *PRPF8* and isogenic-matched control iPSCs lines were generated with CRISPR/Cas9, and all lines were subsequently differentiated to RPE cells. Interestingly, although the differentiated cells were similar to RPE cells *in vivo* (apicobasal polarity and phagocytose photoreceptor outer segments), no phenotypical nor functional differences between the diseases and isogenic

control cells could be observed *in vitro* [87]. This lack of phenotype highlights the need for a more complex cellular model where not only RPE but also photoreceptors can be analyzed together, especially in instances where the identity of the cell type first affected in disease is not clear, as is the case with *PRPF8*. Isogenic-pair iPSC lines have also been generated from a patient with late-onset non-syndromic RP caused by heterozygous mutations in the *CLN3* gene [88] and patients with MAK-associated RP [86] corrected with CRISPR/Cas9. Designed of allele-specific CRISPR guides have been used to silence Pro23His rhodopsin mutation causing dominant RP in iPSCs *in vitro*, and pig retina *in vivo* [86] demonstrating the feasibility of using CRISPR-based genome editing *in vitro* and *in vivo* for allele-specific targeting and correction of autosomal dominant diseases.

A human model of macular dystrophies has been develop using iPSC-RPE cells derived from patients with Doyne honeycomb retinal dystrophy/malattia Leventinese [89]. This model has described some of the key pathological features of macular dystrophies [89]. When compared to controls, the macular dystrophy-diseased RPE showed an increase in drusen-like deposits and in their extracellular accumulation underneath the RPE. Interestingly, the DHRD RPE cells produce drusen-like deposits with a different composition to those generated by the control RPE cells including those derived from CRISPR corrected iPSCs [89].

Usher syndrome is a rare autosomal recessive disease and is the most common inherited form of both visual impairment and hearing loss [90]. Up to 13 genes have been identified as causative of Usher syndrome, with mutations in *USH2A* being identified as the most prevalent gene involved in Usher syndrome and some cases of non-syndromic RP. Fibroblasts from USH2A patients with a heterozygous mutation were corrected using the CRISPR/Cas9 system as a proof of concept [91]. iPSCs have also been generated from USH2A patient's keratinocyte and differentiated into photoreceptor-like cells after transplantation of photoreceptor precursor cells into the retinas of immune-suppressed mice [92]. At this stage, there is no published report of *in vitro* disease models of Usher syndrome using both iPSCs and CRISPR technologies.

Apart from congenital retinal diseases, CRISPR/Cas system and PSCs have also been applied for the development of a human retinoblastoma model [93]. Biallelic inactivation of Retinoblastoma-1 (*RB1*) initiates retinoblastoma, the most common primary intraocular paediatric cancer. CRISPR/Cas9 was used to generate an *RB1*-null hESC line that generated significantly larger teratomas. The teratomas had a dramatic enhancement of neural structures and mitochondrial dysfunction similar to retinoblastoma tumours [93].

One of the limitations of the CRISPR/Cas system is its difficulty to genetically modify mitochondrial DNA. This represents a challenge to generate isogenic controls for the understanding of mitochondrial DNA diseases such as Leber's Hereditary Optic Neuropathy (LHON). LHON is characterized by the loss of retinal ganglion cells (RGCs) resulting in sudden visual loss. Our group used a cybrid approach to replace mitochondrial DNA and generate "isogenic" controls in an iPSCs model of LHON [94]. Cybrid technology was used to replace the mitochondria from LHON patients' fibroblasts with healthy mitochondria from wild-type keratinocytes. iPSCs were generated from LHON and isogenic control fibroblasts and subsequently differentiated into RGCs. Increased basal level of apoptosis was observed in LHON RGCs compared to isogenic cybrid controls indicating that the susceptibility of RGCs death is a direct consequence of the mitochondrial DNA mutations [94].

CRISPR/Cas9 has also been used for the generation of PSC reporter lines specific for RGCs (*BRN3B* in hESCs) and photoreceptors (*cone–rod homeobox* gene in iPSCs) [95, 96] with some advantages over previous knock-in technologies. Traditionally, the fluorescent reporter gene had to include the promoter sequence of the gene of interest or had to be introduced in a specific locus of the gene of interest causing the knock-in lines to become heterozygous. CRISPR/Cas approach does not require the introduction of the promoter of the targeted gene and allows the expression of

an intact target protein and fluorescent reporter protein. This is achieved by inserting the fluorescent reporter gene at the 3′-end of the targeted gene and the addition of a 2A peptide that results in post-transcriptional cleavage of the target protein and fluorescent reporter protein [95, 96].

31.3.3 *In Vivo* and Clinical Application of CRISPR/Cas System

Gene augmentation therapy in humans has already proven to be a promising treatment for different retinal diseases and several clinical trials are ongoing [97]. This is done by the expression of a normal copy of a gene, most commonly with an adeno-associated viral (AAV) vector as a vehicle that can be delivered by subretinal injection [98]. As an example, a preclinical study that optimized a more stable RPGR codon for AAV8 [99] delivery has allowed the beginning of clinical trials for patients with RP (https://clinicaltrials.gov/ct2/show/NCT03252847). However, the augmentation therapy approach is applicable for the treatment of haploinsufficiency or loss-of-function mutations and the expression of the healthy gene is transient. CRISPR/Cas can provide an alternative for the treatment of other monogenic diseases in a permanent manner. The advances in CRISRP/Cas technology and the successful achievement of proof of concept in the generation of animal models and gene therapy *in vivo* and *in vitro* will provide the platform needed for CRISPR-based clinical trials in the eye.

A recent study developed a CRISPR gene editing strategy to remove the aberrant splicing variant of the *CEP290* gene and restore normal protein expression in a humanized mouse and a non-primate model of LCA [100]. The novel system (EDIT-101) used an AAV5 vector to deliver Cas9 and gRNAs to the photoreceptor cells by subretinal injection. The local delivery and the use of the photoreceptor-specific *GRK1* promoter allowed to limit the expression of the CRISPR/Cas9 system to only the therapeutic target tissue and cell type. The study provides a path for the preclinical development of the treatment of patients with CEP290-associated retinal disease [100]. The US Food and Drug Administration has already approved the EDIT-101 CRISPR system for a clinical trial Phase I/II to correct a point mutation in *CEP290* for the treatment of LCA10 patients as mentioned in the news section of Nature Biotechnology (https://www.nature.com/articles/d41587-018-00003-2). Of note, a first time clinical trial for patients with stage IV metastatic non-small cell lung cancer using CRISPR gene editing in humans is in Phase I (https://clinicaltrials.gov/ct2/show/record/NCT02793856). No results have been published yet, though this will be an essential precedent to assess the safety and potential benefits of CRISPR gene editing in humans.

31.4 Summary

Our understanding of the pathogenic processes involved in retinal diseases largely hinges on the provision of biologically relevant models. With the advent of iPSC technology in 2006, it became possible to study patient-tissue *in vitro*, enabling scientists to investigate the genetic determinants of retinal diseases. Since the first papers were published in early 2000 showing that hPSCs could be differentiated into retinal progenitors, there has been a surge in the number of reports showing that retinal development could be mimicked *in vitro*. The discovery of retinal organoids overcame limitations in simple two-dimensional models, leading to the breakthrough that all retinal neurons could be derived from hPSCs. Now, scientists have the necessary tools to accurately model retinal diseases *in vitro*, a prospect that had seemed implausible only 18 years ago. With the harnessing of the gene-editing tool CRISPR, scientists are now in the unprecedented position of unearthing how genetics intersects with disease, providing not only an invaluable tool to improve current disease models but potentially providing real therapies for patients with blinding diseases.

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About the Editors

Dr. Gyan Prakash has a deep-rooted dedication and passion for global health, international research collaborations, and more than 30 years of experience in international health research, teaching, and mentorship. He has worked across many biomedical disciplines including infectious diseases, drug development, eye diseases, substance and drug abuse, oncology, neurology, and biomedical technology. He has served as Director of the Office of International Program Activities (OIPA) at the 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 human and infrastructure capacity, 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) and Johns Hopkins University. In addition to his long-standing industry career and government tenure, Dr. Prakash has served as an Adjunct Professor at the University of Maryland and George Mason University in leading the biotechnology management programs at the universities, mentoring graduate students and teaching.

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 several 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 the President and CEO of Metastatin

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Pharmaceuticals, Inc., a cancer biologics-development stage company in Bethesda.

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 the American Society for Microbiology, and a member on national committee for major medical and professional associations. 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 the 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 to his credit that he published with Marcel Dekker 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, MS and PhD 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 BS (Biology and Chemistry) and an MS (Biochemistry) at the 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 postdoctoral 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 and was featured on *The Frontline* on PBS television. At National Eye Institute-NIH in the USA, Dr. Prakash initiated, designed, and established several international research partnerships, collaborations, and training programs around the world, including the partnership with National Institute of Sensory Organs (NISO) at Tokyo Medical Center in Japan that led to the establishment of Global Eye Genetics Consortium, GEGC (previously named Asian Eye Genetics Consortium, AEGC), for which he served as the founding president in 2014 (www.gegc.org). The GEGC represents over two hundred scientists and clinicians from more than thirty countries. Dr. Prakash has received numerous national and international honors, fellowships, and awards, including NEI Director's Award, Diversity Alliance Science Award, Aditya Jyot Foundation Award, Dr. Brahma Swaroop Oration, and many other honors around the world.

Dr. Takeshi Iwata received his PhD from the Department of Agriculture at Meijo University in Japan and worked at the National Eye Institute (NEI)/National Institutes of Health (NIH) in Bethesda, MD, USA, as a postdoctoral fellow in retina genetic laboratory, headed by Dr. George Inana, MD, PhD. 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 used the OAT cDNA probe to hybridize OAT pseudogenes in X-chromosome and perform linkage analysis for X-linked retinitis pigmentosa families. He moved to Bascom Palmer Eye Institute, University of Miami School of Medicine in Miami, Florida, with Dr. Inana to work on other hereditary retinal diseases. Dr. Iwata returned to the NEI laboratory headed by Dr. Deborah Carper, PhD, to work on another major retinal disease, diabetic retinopathy. His work was to identify the mechanism of transcriptional regulation for two genes, aldose reductase and sorbitol dehydrogenase, in polyol pathway associated with the disease. These retinal and gene-related works 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), National Hospital Organization Tokyo Medical Center.

Dr. Iwata is currently running three main projects related to age-related macular degeneration (AMD), inherited normal tension glaucoma (NTG), and inherited retinal diseases (IRD) 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 et al., Prog Retin Eye Res 2016). In 2020, his group identified a novel gene *MCAT* (Li & Shinqin et al., Hum Mol Genet 2020) as responsible for inherited optic neuropathy and preparing to report a novel gene for inherited glaucoma. His IRD works also identified novel genes including *RP1L1* for occult macular dystrophy Miyake's disease (Akahori et al., Am J Hum Genet, 2010), *CCT2* for Leber congenital amaurosis (Minegishi et al., Sci Rep 2016), *C21orf2* for retinitis pigmentosa and cone-rod dystrophy (Suga et al., IOVS 2016), and *LRRTM4* for macular dystrophy (Kawamura et al., J Hum Genet 2018). In 2011, Dr. Iwata started with a small group of Japanese ophthalmologists specialized in retinal electrophysiology to perform high-quality diagnostic and whole exome analysis of entire family with hereditary retinal diseases. This small group has now expanded to 30 Ophthalmology Departments and Institutions as the Japan Eye Genetics Consortium (JEGC), which became a model for Asian Eye Genetics Consortium (AEGC) in 2014 and Global Eye Genetics Consortium (GEGC) in 2018.

Dr. Iwata has received awards from the Cooperative Cataract Research Group, National Foundation for Longevity Science, Foundation for Preventing Blindness, Japanese Association for Complement Research, Japan Retinitis Pigmentosa Society, International Society for Eye Research (ISER) Special Recognition Award, Dr. A.P.J. Abdul Kalam Award, and Public Endowment Award from Aditya Jyot Eye Hospital and has given keynote lectures around the globe including major universities in the USA, the UK, China, India, and Japan. He is currently the Visiting Professor of the Department of Ophthalmology, Wenzhou Medical University in China; Department of Ophthalmology, He University in China; and Henan Eye Hospital/Institute, Zhengzhou University in China. Dr. Iwata has served as a committee member in a number of local and international organizations including the Association for Research in Vision and Ophthalmology (ARVO), Asia-Pacific Academy of Ophthalmology (APAO), International Society for Eye Research (ISER), and International Council of Ophthalmology

(ICO). He has served as a committee member of the ARVO Foundation Award Committee, ARVO Global Research Training Committee, ARVO Executive Committee Member for the Advocacy Pillar, ARVO Journals Chief-Editor Nominating Committee, APAO Program Committee, ISER Vice-President, and the Program Chair of XXII Biennial ISER Tokyo Meeting in 2016. Dr. Iwata has over 150 publications in scientific journals, reviews, and book chapters. He currently serves on the Editorial Board for *Journal of Ocular Biology, Diseases, and Informatics*, *Eye and Brain*, *Molecular Vision*, *Human Genomics*, *Annals of Eye Science*, and *Asia-Pacific Journal of Ophthalmology*.

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 in Bethesda, USA; Buffalo Niagara Medical Center Campus in Buffalo, USA; Aditya Jyot Eye Hospital in Mumbai, India; Moorfields Eye Hospital-University College in London, UK; Ningxia Eye Hospital, Ningxia People's Hospital in Yinchuan, China; and Henan Eye Hospital in Zhengzhou, China. As the current president of GEGC, Dr. Iwata is actively involved in identifying the key leaders around the world to build effective plans for future genetic eye research programs. Dr. Iwata has worked with Dr. Gyan Prakash since 2013 to build eye genetic research programs around the world. The collaboration has led to the production of three volumes of the book series, ***Advances in Vision Research*** published by Springer Nature.

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