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Takeshi Iwata *Editors*

Advances in Vision Research, Volume I

Genetic Eye Research in Asia and the Pacific

Springer

Essentials in Ophthalmology

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Editors

Advances in Vision Research, Volume I

Genetic Eye Research in Asia and the Pacific

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The book – volume 1 is dedicated to the blind children of the world, their caregivers for the noble cause, and the vision researchers around the world who are finding the solutions to treat the blindness

Foreword

It gives me much pleasure to write an introduction for this interesting, important, and timely book that collects the writings of the leaders in ocular genetic research from throughout the Asian Pacific region. It is a masterful review of the current knowledge and an outline of the future challenges. It is a must read book, and it will be a long-term resource for all those interested and working in this field.

Ocular genetics is a challenging area. Some of the studies of the genetic basis of eye disease have led to major breakthroughs and better understanding for the whole field of genetics; retinoblastoma is only one such example. For some of the less common inherited conditions, a single gene has been successfully identified usually by studying affected families. In other cases, such as retinitis pigmentosa, an essentially similar phenotype has been shown to have more than a hundred different genotypes with still more to find.

However, for some common ocular disorders that clearly have a strong hereditary component such as age-related cataract, age-related macular degeneration, or glaucoma, it has been very hard to identify more than a handful of the genes that play a contributory role. Progress in elucidating the basis for these complex conditions is likely to be made by studying much larger and diverse populations, and that is one of the goals and the strengths of the Asian Eye Genetics Consortium (AEGC) and also the basis for this book.

The AEGC also makes available training and increased access to the exciting new technology to its collaborative network. The AEGC will facilitate the collaborative expansion of ocular genetic research in a different Asian population that represents two thirds of the world people and that until now has been relatively little studied. It is a very exciting initiative that undoubtedly will contribute to our knowledge about inherited eye disease and our ability to manage them.

This book is intended to be a starting point and serves to promote and guide future studies and spur further publications documenting new findings. I feel confident in strongly recommending this important publication to all

those with an interest in ocular genetics and congratulating the authors of a fine piece of work.

International Council of Ophthalmology (ICO) Hugh R. Taylor
San Francisco, CA, USA
University of Melbourne, Melbourne, Australia

Preface

The concept for this book was conceived following a conversation between one of the editors and his 87-year-old mother on the importance of eye health as one ages. The editor's mother mentioned that two key things shaped her typical day: having an alert mind and maintaining strong eyesight. If her mind and her vision were responding well, she could look forward to enjoying her day. This anecdote highlights the vital role eye health plays in ensuring a high quality of life at any age. As the quest for human longevity grows wider and deeper, it is increasingly clear that improving eye health is an important frontier for scientific and clinical research.

There has been rapid proliferation of available information on eye diseases, including results from genetic eye research studies in the western population. Major advances in technology have been incorporated into scientific research on eye disease in the western world. However, more than 60% of the genes involved in eye disease within the Asian population remain unknown. This is due to a variety of reasons, including lack of funding, strategy, facilities, and training of researchers. The lack of information on the genetic basis of eye disease in the Asian population is particularly relevant to new research, as over 90% of the global burden of eye disease is shouldered by developing countries, where many treatable diseases often go undiagnosed. According to the WHO, close to 39 million people around the world are blind, and a further 246 million are not able to see properly. An exact figure on the number of blind in Asia is not available, but it is estimated that 7 out of 10 affected people live in the Asian countries, thus making the Asian continent as the most affected region in the world.

There are new efforts underway to encourage important discussions on the subject, and new plans are being implemented to discover the genes responsible for many eye diseases in the Asian population. It is expected that the results of this work will lead to important discoveries in the genetics of eye diseases and improved understanding of the biology of many eye diseases. Recent progress has been made in international research collaborations between developed and developing countries to address a number of vision research problems. Many of the reasons for causing blindness have been researched, but in several areas of the world, especially in Asia, a coordinated strategy for basic science and health services research is required to further

reduce the global burden of eye diseases. This book represents one of the several steps to begin coordinating the findings from existing research studies on eye disease in Asians, translating those conclusions into real-world clinical solutions, and identifying important areas for further research.

We have the great honor and privilege to highlight this work on research related to the Asian eye diseases. We have assembled more than 100 leading researchers from the fields of human genetics, ophthalmology, molecular biology, biochemistry, sensory sciences, and clinical research to present the current status of the growing field of genetic eye research in Asia. Our hope is that this book will provide a strong background for all researchers, clinicians, clinical researchers, and allied eye health professionals with an interest in eye diseases of the Asian population. 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-ups of their scientific research and findings. As the book editors, we have neither interfered with any presentation nor verified any materials covered in the book chapters and do not assume any responsibilities, direct, indirect, or implied, for the chapter contents in any way or fashion. We were privileged to have worked with a group of authors who are recognized leaders in their respective fields and who willingly gave their time to contribute to this volume despite their busy schedules. We are forever in their debt.

This book would not have become a reality without the support, encouragement, and assistance of several peers and distinguished colleagues. Dr. Deborah Carper, former deputy director of the National Eye Institute at the National Institutes of Health, brought us together and provided the mentorship and inspiration for the collaboration. Toshiro Mikami, Chieko Watanabe, and Hemalatha Gunasekaran from Springer provided the impetus for the initiation and continued support of this project. Dr. Arun Singh of Cleveland Clinic, the series editor, provided the support for inclusion in his acclaimed series. We are very grateful for the valuable support of the leadership, senior management, and many distinguished colleagues of the National Eye Institute in the USA and National Hospital Organization, Tokyo Medical Center in Japan. Finally, and most importantly, we are truly indebted to our family members including Dr. Savita Prakash, Dr. Fumino Iwata, Dr. Shivaani Prakash, and Gary Prakash 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.

As we live longer, there is a greater need to maintain good eye health. New tools and methods are being developed at a fast pace in many parts of the world as human aging has given rise to an accelerated research in the vision-related diseases. The impact of findings from several elegant genetic

research studies is being translated to impact care at the ground level, in hospital laboratories, clinics, and other settings where genetic analyses are being performed. Our hope is that the researchers in the Asian eye disease and vision research community continue to coordinate their efforts as they endeavor to shed light on the genetic and clinical factors that affect eye health.

Bethesda, MD, USA — Gyan Prakash

Tokyo, Japan — Takeshi Iwata

Contents

Asian Eye Genetics Consortium (AEGC)

1

Gyan Prakash, Takeshi Iwata, and S. Natarajan

Abstract

Asian Eye Genetics Consortium (AEGC) was established in 2014 to encourage and focus on eye disease research in Asia. With significant advancements in DNA sequencing and internet data exchange technology, we are experiencing a new era in the field of human genetics. Decades of eye genetic research have shown that genetic variations are widely involved in eye diseases. These genetic involvements can be highly penetrant as in Mendelian eye diseases or highly associated as risk factors in common eye diseases. In both cases, patient genome sequence is quickly determined and traced within family or compared with millions of genome sequences collected around the world and stored in a database. However, most of the information originates from population of European descent, and information on other ethnic groups are limited. AEGC focuses on eye research in Asia, the most populated region of the world where very little has been explored for genetic eye diseases. The new consortium has brought a collective thinking and ideas from researchers around the world who have interest in genetic eye research in the Asian region.

Keywords

AEGC • AIOS • ARVO • Collaboration • Consortium • Data exchange • Human genetics • Mendelian eye diseases • Personalized medicine • SAARC • SAO

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1.1 Introduction

With new and significant advancements in DNA sequencing and internet data exchange technology, we are experiencing a new era in the field of human genetics research. Decades of eye genetic research have shown that genetic variations are deeply involved in eye diseases. These genetic involvements can be highly penetrant as in Mendelian eye diseases or highly associated as risk factors in common eye diseases. In both cases, patient genome sequence is quickly determined and traced within family or compared with millions of genome sequences collected around the world and stored in a database. However, most of the information originates from population of European decent, and information on other ethnic groups are fairly limited. The Asian Eye Genetics Consortium (AEGC) was established to focus on eye disease research in Asia, the most populated region of the world where very little has been explored for genetic eye diseases. The consortium has brought a collective thinking and ideas from the researchers around the world who have interest in genetic eye research in the Asian region.

1.2 The Initial Concept of Asian Eye Genetics Consortium (AEGC)

Dr. Deborah Carper, former Deputy Director, National Eye Institute (NEI-NIH) in the USA, introduced Dr. Gyan Prakash at NEI to Dr. Takeshi Iwata at Tokyo Medical Center (TMC) in 2012 and helped in developing a research collaborating agreement between the two institutions. Subsequently, NEI Director, Dr. Paul Sieving and Dr. Yozo Miyake (on behalf of TMC) signed a research collaboration agreement during the World Ophthalmology Congress in April 2014 (Photo 1.1). The signing of research collaboration between the two institutions began a series of discussions in 2013-2014 leading to the establishment of Asian Eye Genetics Consortium (AEGC).

In 2014, National Institute of Sensory Organs (NISO), Tokyo Medical Center (TMC), and National Hospital Organization (NHO) in Japan developed a Japan Genetic Consortium for Hereditary Retinal Diseases. The initial consortium was established by Dr. Takeshi Iwata, Dr. Kazushige Tsunoda, Dr. Yozo Miyake, Dr. Mineo Kondo, Dr. Takaaki Hayashi, Dr. Kei

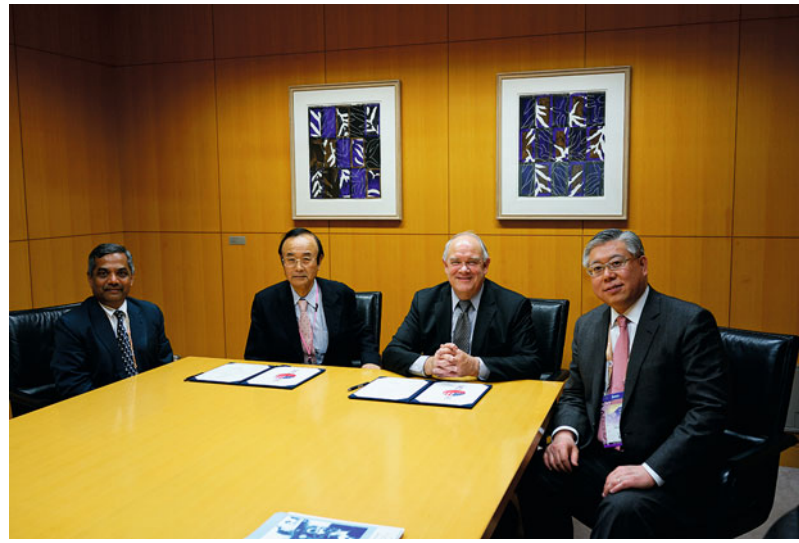

Photo 1.1 NEI Collaboration with NISO-TMC at WOC-2014 in Tokyo

Photo 1.2 Foundation of AEGC at ARVO-2014 in Orlando

Shinoda, and Dr. Kazuki Kuniyoshi to perform whole exome analysis on Japanese patients with inherited retinal diseases to identify disease-causing mutations. The initial study resulted with 85% of affected families with novel mutations leading to the hypothesis that unidentified mutations are dominant in Japanese population with inherited retinal diseases, and this may also be true for other Asian population. The research collaborations with eye institutions in the Asian region that have the facility and research interests in studying eye genetics in the patients with rare diseases became the core focus of the new consortium.

The NEI collaboration with TMC along with utilizing the already established ties with India, China, and other Asian countries resulted in the formal launch of the Asian Eye Genetics Consortium at the annual ARVO meeting in Orlando, USA in May 2014. The key founding members represented the following countries: USA, Japan, India, and China (Photo 1.2). Dr. Fielding Hejtmancik (USA) joined the AEGC launch meeting on the phone.

1.3 The Goals of AEGC

The aim of the AEGC is to explore and conduct eye genetics research in the Asian population. Approximately one hundred eye researchers from the Asian countries are currently interacting and collaborating to develop programs to share, catalog, and collaboratively work to identify the genetic aspect of eye diseases in the Asian countries. AEGC has the following goals and plans:

(a) Share genetic information in the Asian population to rapidly isolate common disease-associated variants.
(b) Establish system for accurate diagnosis and grouping of Asian eye diseases.
(c) Establish system for cost-effective genetic analysis.
(d) Develop a research-oriented database to collect, diagnose, and catalog eye diseases in Asia.
(e) Support and foster collaboration among the Asian countries for the advancement of research that will provide genetic information in the Asian population.
(f) Collaborate with other international or regional organizations with similar goals, and
(g) Organize and hold regional congresses and other educational and scientific activities to promote goals of the consortium.

1.4 AEGC Activities in Asian Countries

The AEGC team has accomplished the following milestones since its inception in May 2014, the first AEGC meeting at Association for Research in Vision and Ophthalmology (ARVO) 2014 in Orlando, Florida.

In October 2014, the AEGC conducted a South Asian launch of the program at the 12th Congress of the South Asian Association for Regional Cooperation (SAARC) countries Academy of Ophthalmology popularily known as SAO in Colombo, Sri Lanka. It was a gathering of ophthalmologists and researchers from eight Asian countries (Photo 1.3).

Subsequently, in February 2015, India became the first country to develop an AEGC local country chapter. The Indian chapter was launched at the meeting of All India Ophthalmological Society (AIOS) in New Delhi, India, in 2015. A number of local vision research leaders joined hands to organize the AEGC programs in India and the neighboring Asian countries (Photo 1.4).

Later in 2015, there were exclusive AEGC sessions planned at Asia-ARVO in Yokohama, Japan, in February 2015 (Photo 1.5), and the first

Photo 1.3 AEGC Launch at SAO-2014 in Colombo

Photo 1.4 Launch of AEGC India chapter at AIOS-2015 in New Delhi

Photo 1.5 AEGC Meeting at Asia-ARVO-2015 in Yokohama

Photo 1.6 AEGC Meeting at Tokyo Medical Center in 2015, Tokyo

AEGC database discussion meeting was held at the TMC (Photo 1.6).

The second AEGC annual meeting was held during ARVO 2015 at Denver, Colorado (Photo 1.7). At this meeting, 35 members gathered to discuss the type of possible collaborations by this consortium and planned for publication of a book on "Genetic Eye Research in Asia and the Pacific" by Springer. The meeting introduced and discussed the topic of government and local restrictions for some countries on the exchange of DNA samples.

The ASEAN Nation's Ophthalmology conference in Hanoi, Vietnam, was held in September 2015 (Photo 1.8). There have been a number of vision researchers and community leaders that have led, supported, and nurtured the AEGC organization since its inception in 2014.

Photo 1.7 AEGC Meeting at ARVO-2015 in Denver

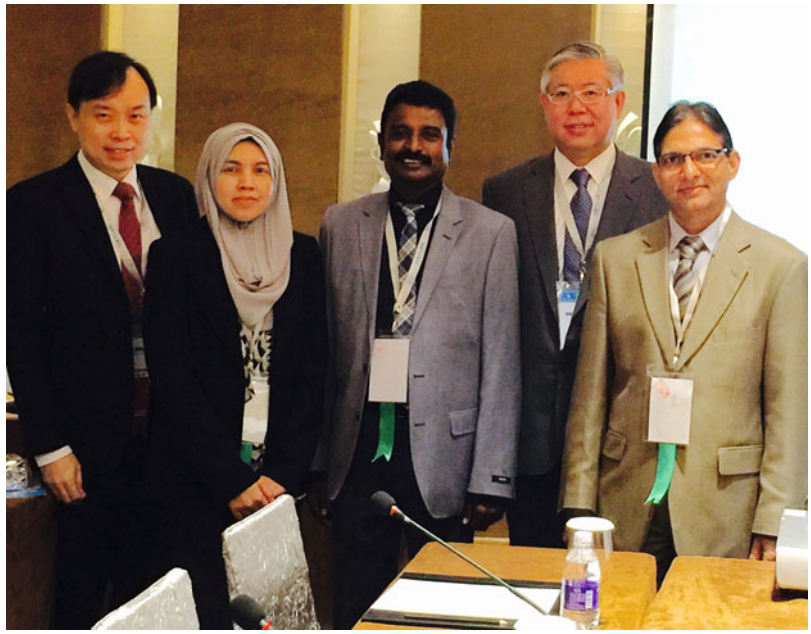

Photo 1.8 AEGC Meeting at ASEAN-2015 in Hanoi

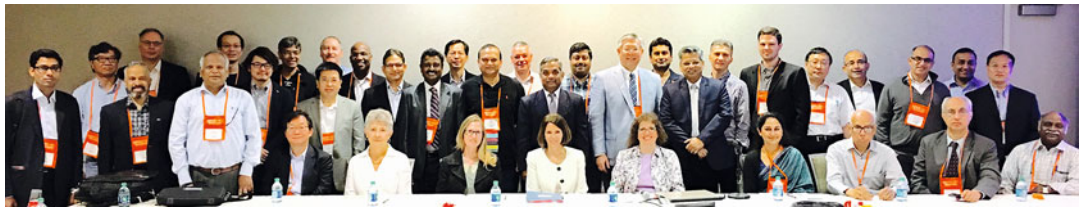

Photo 1.9 AEGC Meeting at ARVO-2016 in Seattle

During the ARVO 2016 meeting at Seattle, over 45 members from 14 countries gathered for the third annual meeting of AEGC (Photo 1.9). At this meeting, the past activities were reviewed and the new plans for AEGC were introduced. The new plan included development of a common genotype-phenotype database for AEGC and submission of funding proposals in each country were suggested. The meeting participants clearly felt that the AEGC had moved to the next phase.

1.5 Growing AEGC Members and Researcher Exchange Programs

Several representatives from the following countries are currently on board and actively participating: US, Japan, India, Sri Lanka, Australia, China, S. Korea, Thailand, Singapore, Malaysia, Indonesia, UAE, Turkey, Bangladesh, and Taiwan. The future representation is being planned in other Southeast Asian countries. Currently, about one hundred and fifty members are part of the AEGC and the number is growing rapidly.

Researcher Exchange Program in the last two years included Dr. Yuriko Minegishi (Japan) going to NEI-USA, Dr. Huiping Li (China) and Dr. Yang Liu (China) to TMC-Japan, Dr. William Sultan (USA) to TMC-Japan, Dr. Fielding Hejtmancik (USA) to TMC-Japan, and Dr. Brian Rossmiller (USA) to TMC-Japan.

A grant approved by the Japan Intractable Disease Research Foundation in 2014 for a senior US researcher was awarded to Dr. Fielding Hejtmancik (NEI-USA) to attend the AEGC session at Asia-ARVO 2015 and visit several institutions in Japan to expand the research collaborations between the US and Japanese eye geneticists.

An International Council of Ophthalmology (ICO) Fellowship center was recently established at Sadguru Netra Chikitsalaya and Eye Institute, Chitrakoot, India. A fellowship in Ophthalmic Genetics is being established at Shroff Eye Hospital in New Delhi, India, in collaboration with Dept. of Human Genetics at Guru Nanak Dev University, Amritsar, India. Under the AEGC collaboration, the two institutions have made commitment to share clinical specimens and conduct genetic eye research. Recently, Dr. Shailja Tibrewal from Shroff Eye Hospital completed a month-long NIH fellowship in 2016 to learn and participate in an international genomic summit at NIH and became the first recipient Ophthalmologist of the prestigious NIH fellowship for the genetics training. Aditya Jyot Foundation for Twinkling Little Eyes in Mumbai, India, recently inaugurated an Eye Genetics laboratory to carry out the AEGC program studies. As an AEGC training fellow, Ms. Sidhita Nare from Aditya Jyot Foundation received training in eye genetics in 2016 at the Tokyo Medical Center. The current President of AEGC, Dr. Takeshi Iwata delivered the first Dr. A.P.J. Kalam Public Endowment lecture on "personalized Medicine and AMD" and discussed the AEGC plans in Mumbai, India in December, 2016. The scholars and visitors programs combined with laboratory- and clinic-based training between the participating institutions are a hallmark of the AEGC goals. Such programs are establishing new eye genetics laboratories in the Asian region and training the interested researchers in the art and science of the eye genetics research.

The AEGC website (http://www.aegc.asia) was launched on November 11, 2015. In March 2016, AEGC invited Prof. Paul Baird, Head of Ocular Genetics at Center for Eye Research Australia, Melbourne, Australia, to lead the AEGC collaborative research programs and develop plan for a common database platform for the AEGC members to use.

1.6 AEGC Database Construction

Based on a survey, the AEGC determined that many Asian countries, including China and India, were prohibited to export DNA samples. The AEGC is developing plans and focusing on sharing genetic information with the researchers in these countries. It will help in establishing classical collaboration of specimen sharing and data analyses for the other Asian countries. The data sharing is being planned by constructing a common database for AEGC to pool genotype-phenotype information.

The AEGC is planning to include the following information in the database: age, sex, gene, gene mutation, disease name, phenotype characteristic (fundus photo, optical coherence tomography, fluorescence angiogram, electroretinogram, adaptive optics retinal imaging, auto fluorescence, etc.), country, ethnic group, and other familial

information. These detail phenotypic characteristics for each gene mutations should be useful in developing useful products for diagnostics or therapeutics and searching for genetic-clinical datasets.

1.7 Summary

The overall goal of the AEGC is to keep the global aspect for genetic eye research and development interconnected thus accelerating the growth of international research collaborations in eye diseases for the advancement of high quality science. The Asian region is experiencing a strong growth in vision research programs and is expected to play a significant role in developing scientific programs in the coming decade. There are more new researchers entering the field of vision research, and new labs are opening doors to adapt new technologies for genetic research in the Asian countries than any other region in the world. At this stage, more than 60% of genes involved in eye diseases are very likely not known in the Asian population. A number of leading labs in the USA and Europe have increasing interests in undertaking new genetic research programs to uncover the biology of eye diseases in the Asian population. A concerted global effort like AEGC will accelerate the collaborative genetic eye research in generating useful new scientific data. The AEGC will seek to uncover new scientific opportunities and identify shared priorities to create unique international opportunities in genetic eye research. The AEGC has an opportunity to help in establishing partnerships among scientists, governments, companies, and non-government organizations to leverage research programs for human health and understanding of the biology of eye diseases. The underlying principles of the AEGC collaborations are to conduct research work with the highest scientific quality and ethical standards. The AEGC members have shared goals and interests. There is also a local relevance for the vision research collaborations. The AEGC is working with the local researchers and government leaders for the mutual benefits in developing international collaborations that will benefit the vision research community around the world.

Japan Eye Genetics Consortium (JEGC) for Hereditary Retinal Diseases

2

Takeshi Iwata

Abstract

Hereditary retinal diseases are groups of eye diseases affecting development, cellular function, or structure of the retina. Millions of patients affected by this diseases worldwide are inherited with gene mutation in autosomal dominant, autosomal recessive, X-linked, or sporadic forms. The phenotype of disease would appear as retina specific or with syndromic complication. Over 255 genes have been reported as disease causing in over 36 retinal diseases. However, the prevalence of these gene mutations often differs between ethnic groups. Over the years, significant progress has been made for patients of European descendants mainly the Caucasians. However, Japanese patients living at the far east of Uracil continent have not fully benefited from these research. In 2014, a Japanese consortium of over 26 universities, institutions, and ophthalmic clinics was established to analyze the entire Japanese population with hereditary retinal diseases and to create diagnostic system, genotype-phenotype database, functional analysis of mutant protein, development of mutant animal models, and bridging with industry for therapeutic developments. This chapter is a brief introduction of the history, structure, and current activity of the consortium, and other chapters of this book by member of the consortium will describe the results of each disease in detail.

Keywords

Retina • Gene • Mutation • Hereditary disease • Japanese • Whole exome analysis • Animal model • iPS cells

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2.1 Introduction

Hereditary retinal diseases include over 36 various eye diseases with moderate to severe damage to the retina or elsewhere in the body. These eye diseases follow into several groups of characteristic phenotypes when examined by fundus photograph, optical coherence tomography, electroretinography, fluorescein angiography, and other diagnostic methods. The accuracy of these diagnostic tools and experience is critical for characterization and grouping of similar disease before proceeding to any type of gene sequencing such as the whole genome sequencing (WGS), whole exome sequencing (WES), targeted exome sequencing (TES), or classical direct sequencing. Gene mutations highly associated with hereditary retinal diseases have been extensively studied in the United States and few European countries leading to identification of approximately 255 disease-causing genes [1]. The number of gene mutations are now approximately 12,000 [2]. As more research groups are involved in this field, novel mutations within known and unknown genes are being discovered every month.

Number reports for gene mutation analysis on hereditary retinal diseases in Japanese population have been published mainly by direct sequencing and few using the exon capture and the next-generation sequencing technology [3, 4]. In 2010 our lab identified a novel gene mutation *RP1L1* gene mutation associated with occult macular dystrophy (OMD) (Miyake's Disease), a phenotypically unique retinal disease, which can only be detected by focal ERG of the macula [5–7]. SNP linkage analysis and direct sequencing was performed to identify in more than five pedigrees. This was the first gene identified by a Japanese research group alone, which the disease was also discovered by Japanese ophthalmologist. This success brought attention to the ophthalmology field in Japan for further investigation of hereditary retinal disease in Japanese population (Fig. 2.1).

In this chapter, establishment of the Japanese Eye Genetics Consortium (JEGC, http://jegc.jp) for hereditary retinal disease is introduced, and the detailed explanation of specific disease type in Japanese population is given in other chapters by the member of the consortium. The aim, member, structure, flow of diagnostic, computer network system for patient registration and database, and mutant functional studies are described in this chapter.

2.2 Short History of the Consortium

In 2011, a small group was established by six ophthalmology departments, RIKEN, National Institute of Genetics, and Tokyo Medical Center to study the genetics of retinal diseases in Japanese population. This study goup was funded by the Japanese Ministry of Health, Labor, and Welfare to explore the prevalence of published mutations in the Japanese population with hereditary retinal diseases. Collaboration of ophthalmologists with diagnostic skills and researcher with next-generation sequencing technology gathered to perform whole exome analysis of one patient from one pedigree in six retinal diseases. To our surprise, less than 15% of the pedigrees were detected with published mutations [8–22]. This led us in 2012 to switch from patient-only recruitment to the entire family for the whole exome analysis, leading to the identification of novel mutation candidates. In 2014, the consortium grew to the current size of 26 ophthalmology departments by strong support of board member of the Japanese Society for Clinical Electrophysiology of Vision (President, Prof. Masayuki Horiguchi) (Table 2.1).

2.3 Disease, Diagnostic, and Disease Leader

Member of the consortium periodically met to discuss the disease type and the priority of the analysis based on the amount of genetic information available for the Japanese population. The selected 36 diseases are (1) Leber congenital amaurosis and early onset RP (diagnosis $<$ 10 yrs), (2) retinitis pigmentosa (RP), (3) enhanced

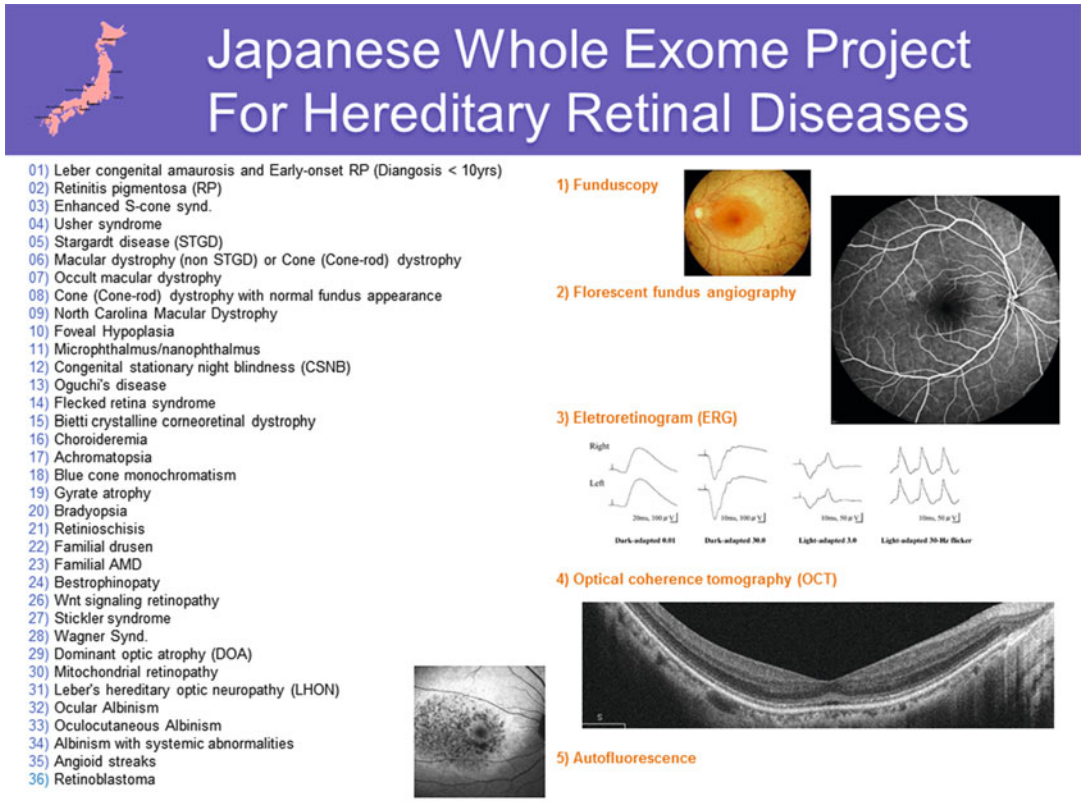

Fig. 2.1 Targeted hereditary retinal diseases and diagnostic information

S-cone syndrome, (4) Usher syndrome, (5) Stargardt disease (STGD), (6) macular dystrophy (non-STGD) or cone (cone-rod) dystrophy, (7) occult macular dystrophy, (8) cone (cone-rod) dystrophy with normal fundus appearance, (9) North Carolina macular dystrophy, (10) foveal hypoplasia, (11) microphthalmus/nanophthalmus, (12) congenital stationary night blindness (CSNB), (13) Oguchi's disease, (14) Flecked retina syndrome, (15) Bietti crystalline corneoretinal dystrophy, (16) choroideremia, (17) achromatopsia, (18) Blue cone monochromatism, (19) gyrate atrophy, (20) bradyopsia, (21) retinoschisis, (22) familial drusen, (23) familial AMD, (24) bestrophinopathy, (26) Wnt signaling retinopathy, (27) Stickler syndrome, (28) Wagner syndrome, (29) dominant optic atrophy (DOA), (30) mitochondrial retinopathy, (31) Leber's hereditary optic neuropathy (LHON), (32) ocular albinism, (33) oculocutaneous albinism, (34) albinism with systemic abnormalities, (35) angioid streaks, and (36) retinoblastoma. All 36 diseases (Fig. 2.2) were selected by this consortium to collect information on patient's phenotype, family information, and the blood or saliva samples for DNA extraction. The consortium came to an agreement that the phenotypic information was critical for identifying and grouping the diseases with common characteristic. To keep high quality of diagnostic information such as fundus photo, fluorescence angiogram, autofluorescence, optical conference tomography (OCT), electroretinogram (ERG), and the pedigree information, "Disease Leader" was appointed to several ophthalmologists who are well experienced with the diagnostic of the disease and genetic analysis. This Disease Leader

Table 2.1 Member of the Japan Eye Genetics Consortium (JEGC) for Hereditary Retinal Diseases

Japan Eye Genetics Consortium for Inherited Retinal Diseases

Takeshi Iwata	Tokyo Medical Center	Director
Yozo Miyake	Aichi Medical University	President
Kazushige Tsunoda	Tokyo Medical Center	Director
Masayuki Horiguchi	Fujita Health University	Professor
Shuichi Yamamoto	Chiba University	Professor
Hiroko Terasaki, Shinji Ueno	Nagoya University	Professor
Manami Kuze	Mie Medical Center	Head Physician
Atsuhi Mizota	Teikyo University	Professor
Nobuhisa Naoi	Miyazaki University	Professor
Shigeki Machida	Dokkyo University	Professor
Yoshiaki Shimada	Fujita Health University	Associate Professor
Makoto Nakmura	Kobe University	Professor
Takashi Fujikado	Osaka University	Professor
Yoshihiro Hotta	Hamamatsu Medical University	Professor
Mineo Kondo	Mie University	Professor
Akira Murakami	Juntendo University	Professor
Kazuki Kuniyoshi	Kinki University	Assistant Professor
Kei Shinoda	Saitama Medical University	Professor
Takaaki Hayashi	Jikei University	Assistant Professor
Masayo Takahashi	RIKEN (Kobe)	Project Leader
Kazuo Tsubota	Keio University	Professor
Kiyofumi Mochizuki	Gifu University	Associate Professor
Shuhei Kameya	Nippon Medical University	Associate Professor
Hiroyuki Kondo	University of Occupational and Environmental University	Professor

will check the incoming phenotypic data from all the member of the consortium and decide whether the information is enough to make diagnostic decision. Until the Disease Leader is comfortable with the data and give final approval of the diagnostic decision, DNA sample will not be processed for whole exome sequencing. Twelve Disease Leaders were selected for this consortium to cover 36 hereditary retinal diseases (Fig. 2.3).

2.4 Structure of Sample and Data Flow of the Consortium

The National Institute of Sensory Organs (NISO), Tokyo Medical Center, National Hospital Organization will take responsibility to organized and centralize the flow of data for all member to view their samples in progress (Fig. 2.4). Sample collection of 7 ml whole blood in EDTA tube or 2 ml of saliva in collecting kit (Oragene DNA) are used. The whole blood are outsourced for DNA extraction, while DNA extraction for saliva is done in house at NISO. All DNA with fixed concentration are stored in NISO at 4 degree in TE buffer or −20 degree in ethanol. DNA extracted from blood and saliva equally gives satisfactory quality of sequence data. The DNA sample is outsourced for whole exome sequencing. After testing several exon capture kits, we've decided to use Agilent SureSelect Ver. 3–6 for the 12,000 DNA samples we've collected so far. DNA sequencing was performed by Illumina Hiseq2000, Hiseq2500, and Hiseq4000 sequence at average of 100 reads. The FastQ files generated are transferred to NISO for sequence analysis (Fig. 2.5).

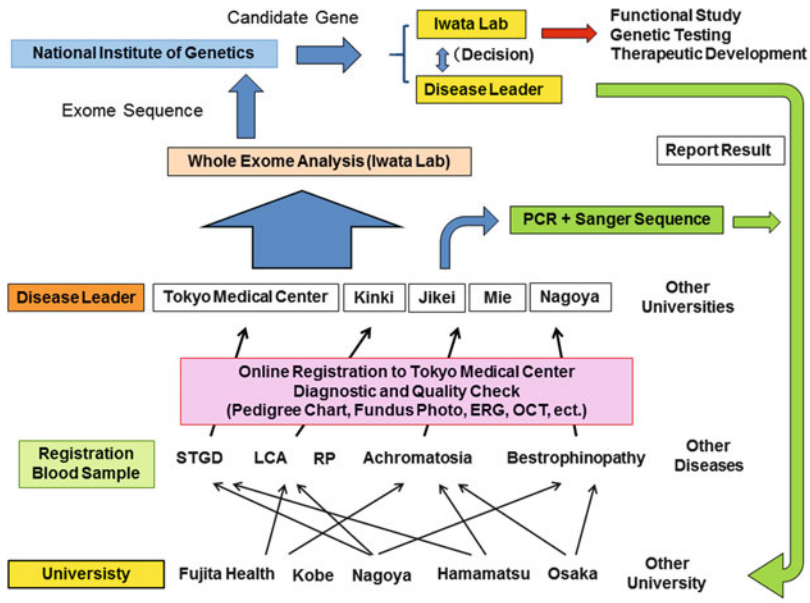

Fig. 2.2 The flow chart of diagnostic decision and genetics analysis

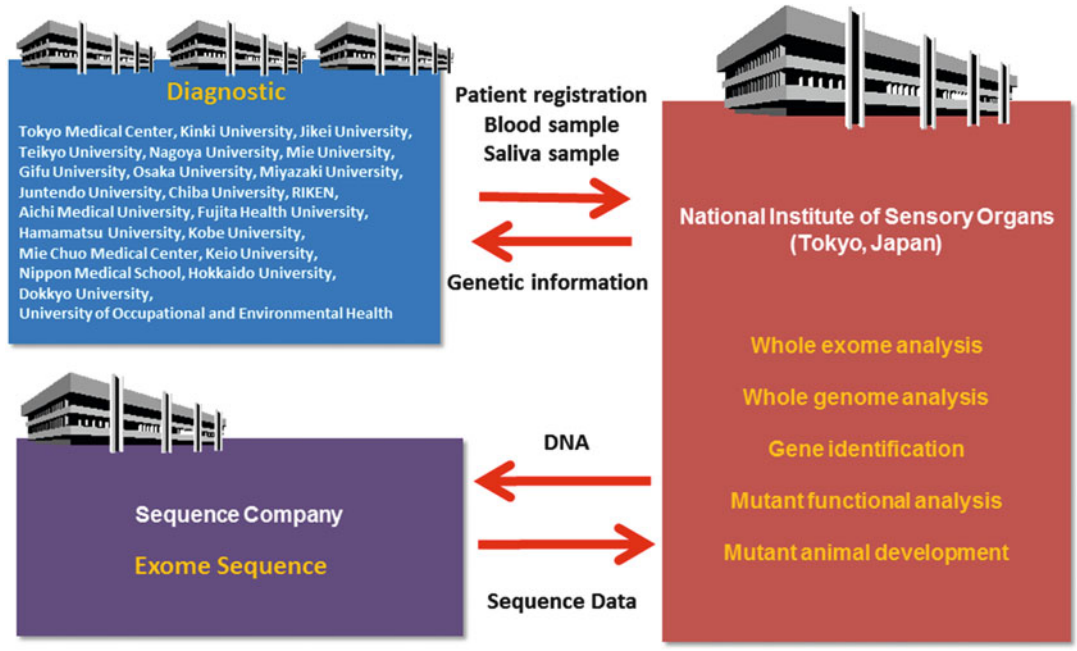

Fig. 2.3 Structure of the consortium and flow of information

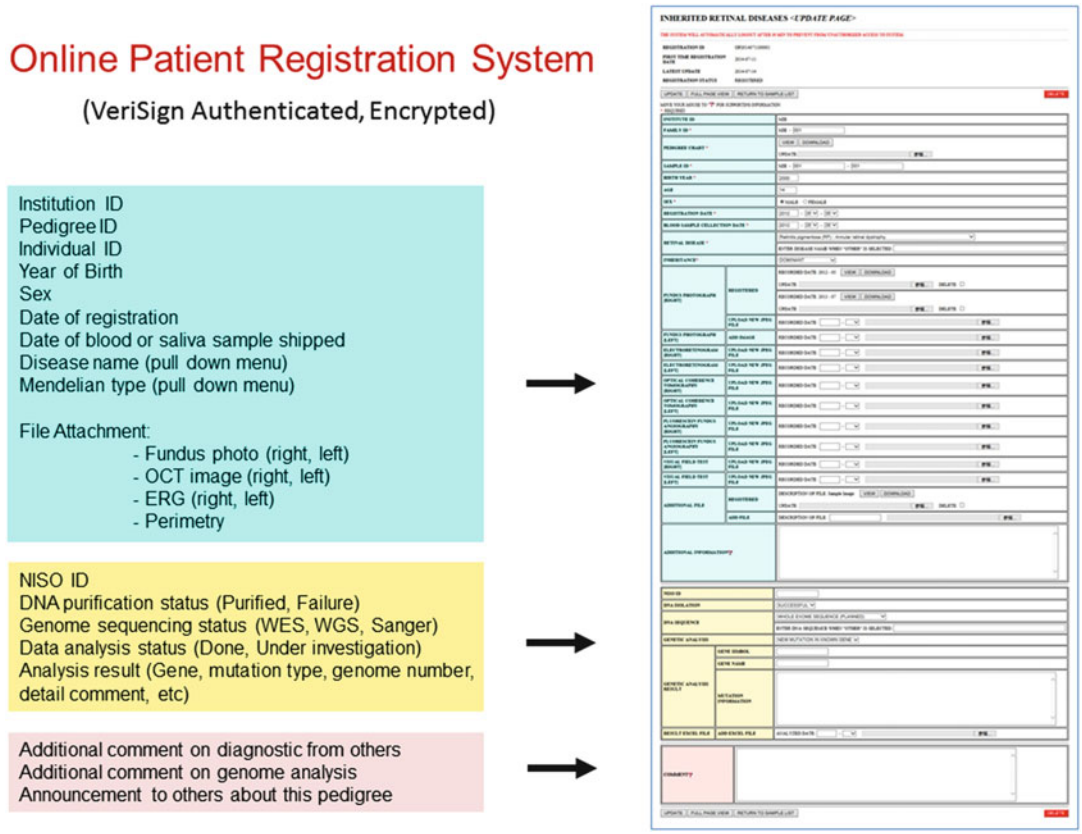

Fig. 2.4 The online patient registration system developed at NISO for collecting phenotype and genotype information

2.5 Online Patient Registration System

Online patient registration system was developed at NISO for all member of the consortium to share and characterize phenotypic and genetic data for each pedigree. It also reduces significant amount of email exchange for the entire project. By entering the system, individual member can easily access to the clinical files for individual fundus photo, fluorescence angiogram, autofluorescence, OCT, and ERG at each date of the examination. Pedigree information and ongoing genetic analysis by whole exome sequencing, targeted exome sequencing, direct sequencing, or other type of sequence detection method can be observed. Any comment can be placed to all or specific member of the consortium. The system is constructed by two separate servers for data input and data storage. The data input is performed by a webserver design with specific segments for clinical data input, genetic data input, and open discussion. The data is encrypted and stored in the separate database server with daily data backup. Significant effort of computer programing was put in to the system for user to easily reach the most likely parameter during the data input procedure to reduce user's time required for input.

2.6 Collected Pedigree by Disease Type

As of 2016, this consortium collected 715 pedigrees plus approximately 600 independent patients for retinitis pigmentosa (RP), Leber

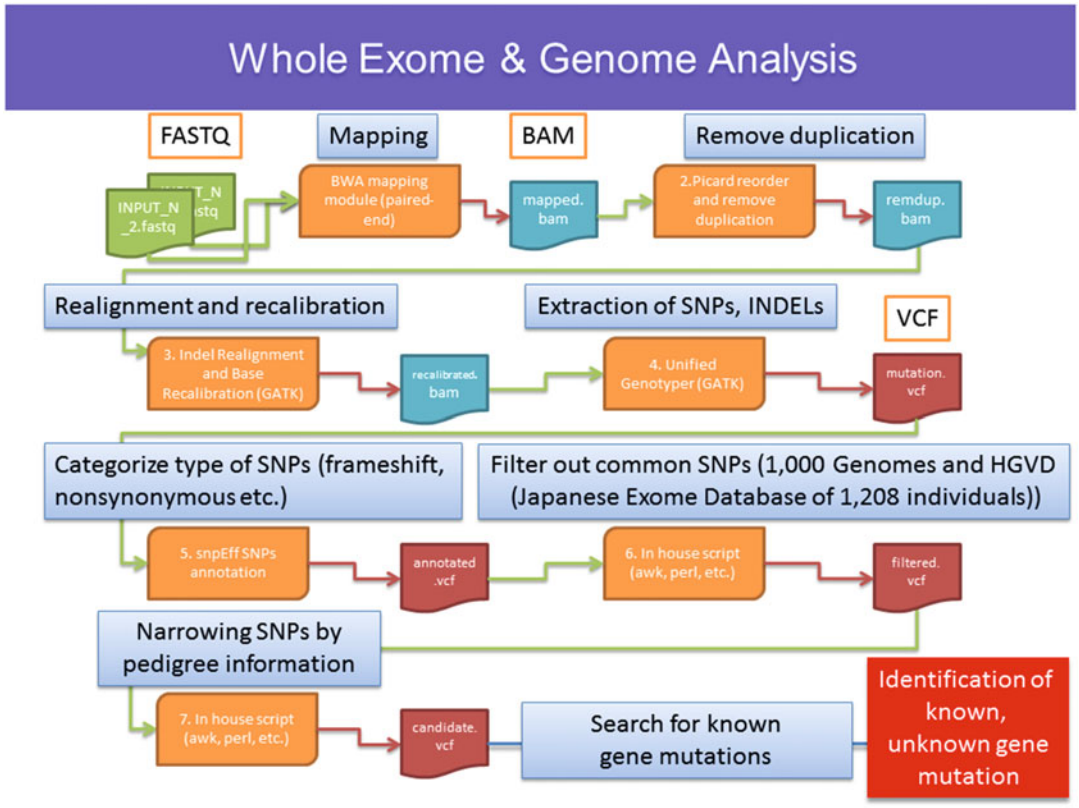

Fig. 2.5 Pipeline of the whole exome sequence analysis

congenital amaurosis (LCA), and macular dystrophy as the major disease. Over 30,000 RP patients are predicted in Japan. Occult macular dystrophy (OMD) and other type of macular dystrophy were selectively collected, and the priority for DNA sequencing of the Leber Congenital Amaurosis pedigrees was set at the highest due to lack of genetic information for the Japanese population. As of 2015, half of the collected 13,000 DNA samples were whole exome sequenced and proceeded for genetic analysis (Fig. 2.6 and Table 2.2).

2.7 Whole Exome Analysis

Reads from the FastQ files were mapped to the reference human genome (1000 genomes, phase 2 reference, hs37d5) with the Burrows-Wheeler Aligner software, version 0.7.10[14]. Duplicated reads were then removed by Picard MarkDuplicates module version 1.129, and mapped reads around insertion-deletion polymorphisms (INDELs) were realigned using the Genome Analysis Toolkit (GATK) version 3.3-0[15]. Base-quality scores were recalibrated using GATK. The calling of mutations was performed using the GATK HaplotypeCaller module, and the called single-nucleotide variants and INDELs were annotated with the snpEff software, version 4.1B,[16] and the ANNOVAR software, version 2015-04-24[17]. The mutations were annotated with the snpEff score ("HIGH," "MODERATE," or "LOW") and with the allele frequency in the 1000 genomes database, Exome Aggregation Consortium (ExAC) database, and HGVD (http://www.genome.med.kyoto-u.ac.jp/SnpDB/index.html). The mutations were then

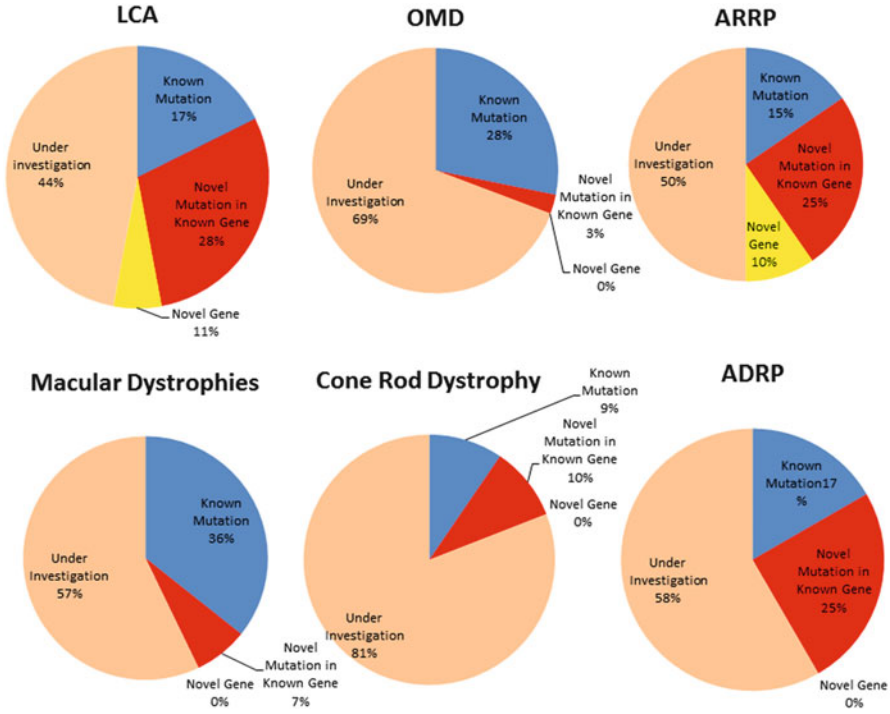

Fig. 2.6 Current status of the whole exome analysis by disease type

filtered so that only those with "HIGH" or "MODERATE" snpEff scores indicating that the amino acid sequence would be functionally affected and with frequency $< 0.1\%$ in the 1000 genomes database, ExAC database, HGVD, and the in-house database of 610 people exome data were further analyzed. We also used new variations which were not found in the in-house exome data of 14 people without ocular diseases. Mutations were filtered by hereditary information in the family members.

The number candidate mutations identified by WES will depend on the inheritance type, pedigree size, and number of affected individual in the analyzed family. From our experience these candidates can be from one to several dozens for each pedigree analyzed. The most likely candidate is selected by frequency of nucleotide change in normal population, the severity of the amino acid change, and whether the gene is expressed in the retina. For dominant pedigree the frequency among normal population should be close 0. The amino acid changes were evaluated by PolyPhen2 or Sift programs and the retinal expression by number of available website information. However, until several pedigrees are identified with the same phenotype and gene mutations, which can be significantly rare to come by, the in vitro and in vivo experiments of the mutant protein is necessary for the proof.

2.8 Result of the Genetic Analysis of the Japanese Population

Overall identification of the known gene mutation was 17%. Novel mutation in known gene was 14% and the novel genes identified as candidate was 10%. The rest of the pedigree lacked samples from part the family, and the whole exome analysis is incomplete. To our surprise, even at this ongoing stage, the

Table 2.2 Collection of DNA samples

Collected DNA Samples	
Disease	**Pedigree**
Retinitis Pigmentosa (AR, AD, XL, SP)	271
Occult Macular Dystrophy	88
Cone Dystrophy	61
Macular Dystrophy	56
Stargardt's Disease	43
Leber Congenital Amaurosis	27
Stationary Night Blindness	21
Familial Dominant Drusen	3
Choroid Dystrophy	6
Circinate Retinopathy	3
Vitelliform macular dystrophy	3
Others	133
Total	715

results clearly show that the majority of the Japanese patients are affected by novel gene mutations.

2.9 Construction of Patient-Derived iPS Cells and Gene Knock-In Animal Models

The novel candidate mutation requires proof of information from the in vitro and in vivo experiments of the mutant protein. One critical information would be the localization of the mRNA or the protein in the retina. In situ hybridization or immunostaining is performed to localize mRNA or protein, respectively, to photoreceptor, retinal pigment epithelial cells, bipolar cells, horizontal cells, or any cells interacting with photoreceptor. Another critical information would be an evidence of behavioral difference between the wild type and mutant protein. This can simply be obtained by transfecting the mutant and the wild-type cDNA into the cells for any change of behavior such as localization, cell shape, cell growth, or cell death.

In this consortium, iPS cells are generated from patients with novel mutation. These cells would be properly used for future experiments when retina can be grown in vitro and analyzed. The technology for generating gene knock-in mouse has advanced significantly these past 2 years with the CRISPR/Cas9 system. The recent modified version of this system has let us to generate a knock-in mouse within weeks. Few gene mutations in our consortium have successfully generated a knock-in mouse and currently periodically examining the fundus photo, OCT, and ERG for any abnormal change in the structure or the function of the retina.

2.10 Development of Genotype-Phenotype Database

One of the objective of the consortium is to create a database for novel and known mutations identified in this study. This may be unique to the Japanese population and may not appear on other database. A genotype-phenotype database describing the detail phenotypic characteristics of each mutation by different individual at different age and time of recording should inspire researcher who can only access to a poor resolution image in the publish paper. With the ID and password, raw data straight from the diagnostic device can be observed at this database. Each selective patient dataset of genotype and phenotype will be transferred to public genotype-phenotype database from patient registration database, which will communicate with the web server for public access. The data will be limited to information, such as year of birth, pedigree, gene, mutation, fundus photo, fluorescence angiogram, autofluorescence, OCT, and ERG on one continuous web page. This genotype-phenotype database is planned for completion in 2016 at http://www.eye.go.jp.

2.11 Discussion and Future Prospects

The advancement in exon capturing and DNA sequencing has led us to perform whole exome analysis of the entire family and analyze all genes to identify disease-causing mutation. The amount of information insured by this genetic analysis has encourage ophthalmologists to ask patients to participate the research project. We have now competed the system flow of diagnostic, patient registration, genetic analysis, and mutant functional analysis and moving on to the development of genotype-phenotype database with easy browsing capability. Tablet based is currently in consideration to search by gene name or by disease characteristic to select mutation, which will display various phenotypic data of patients by finger or pen navigation. This web server will be placed separately from the current patient registration systems.

We are also in production of an automated genotyping system using the LUMINEX protocol distributed by Medical & Biological Laboratories Co., LTD in Japan. A bead probe specifically designed to hybridize to a specific mutation will be used. Multiplex PCR will amplify 30–40 targeted gene mutations simultaneously. All mutations identified by this consortium will be ready for genotyping by year 2016. We expect this system to be incorporated into the commercial service for future use when this consortium project is over.

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Whole Genome Sequencing in Genetic Eye Diseases

3

Zi-Bing Jin

Abstract

Tremendous efforts have been made to develop comprehensive molecular diagnosis for genetic eye diseases in the past two decades. The advent of next-generation sequencing (NGS) has revolutionized genetic diagnosis. Whole genome sequencing (WGS), which allows for the sequencing of an entire genome, is a robust technique that can be used to identify genetic determinants indicative of genetic eye diseases. With increased speed and decreased costs, WGS will become a valuable tool in research and routine molecular diagnosis. Herein, we review the advantages, procedures, applications, and challenges of using WGS in molecular analysis of genetic eye diseases.

Keywords

Whole genome sequencing • Genetic eye disease • Molecular diagnosis • Inherited retinal dystrophy • Next-generation sequencing

3.1 Introduction

Genetic eye diseases contribute to a significant portion of vision impairment and global blindness worldwide, ranging in prevalence from common disorders to specific subtypes of rare conditions [1]. There are many different types of monogenic ocular disorders such as retinitis pigmentosa, microphthalmia, or syndromic eye disorders like Usher syndrome and Bardet-Biedl syndrome [2–5]. Meanwhile, recent studies have demonstrated that the genetic basis played very important roles in the pathogenesis of complex ocular diseases such as age-related macular degeneration (AMD) and primary open angle glaucoma (POAG) [6, 7]. Because of the enormous heterogeneity in genetic etiologies and clinical manifestations, accurate molecular diagnosis of patients with genetic eye diseases has proved to be very challenging for ophthalmologists and geneticists [8]. Traditional technologies such as Sanger sequencing are

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limited in efficiency and power because using them to analyze a target sequence is not only time consuming, but also labor intensive. The costs of Sanger sequencing of a large gene such as USH2A and ABCA4 are no less than those high-throughput techniques [9]. Another previously popular method, array-based primer extension (APEX) genotyping microarray chips, also has limited detection power. Furthermore, microarray-based screening is only designed to uncover known mutations and is not applicable for detecting novel variants [10]. Therefore, application of these traditional technologies in molecular diagnosis of genetic eye diseases is limited by the low mean effective detection rate or by the relatively small number of testable target regions.

The advent of next-generation sequencing (NGS) techniques has greatly improved the molecular diagnosis and identification of disease-causing genes in genetic disorders [11–13]. NGS is a powerful approach that allows for the simultaneous detection of multiple genes, which is particularly crucial in molecular diagnosis of inherited eye diseases [14, 15]. Next-generation sequencing (NGS)-based methods include three strategies: whole genome sequencing (WGS), whole exome sequencing (WES), and targeted exome sequencing (TES). Most of the previous studies regarding genetic eye diseases relied on WES or TES for the identification of specific genetic defects, while WGS was not considered as a common method due to its high cost. However, with the rapid decrease in costs and distinctive advantages, WGS is gradually starting to play significant roles in analyses of genetic components in various genetic eye diseases [16]. This review focuses on the advantages, procedures, applications, and challenges of using whole genome sequencing in molecular analyses of genetic eye diseases.

3.2 Advantages of Whole Genome Sequencing

The evolution of next-generation sequencing (NGS), also known as massive parallel sequencing, has significantly improved the identification of genetic causes in both rare Mendelian diseases and common heterogeneous disorders [17–19]. Next-generation sequencing allows for the simultaneous sequencing of a large number of genes in a single assay. In addition, NGS can be implemented on selected target genes or on the whole genome based on the strategy employed: (1) whole genome sequencing (WGS), (2) whole exome sequencing (WES), and (3) targeted exome sequencing (TES). TES is performed on a custom panel, which includes selected and possible candidate causative genes. It has been proven useful and efficient for identifying pathogenic mutations in patients with [20]. However, TES is hindered by its inability of uncovering new disease-causing genes [21]. As the number of newly revealed causative genes rapidly increases due to the application of next-generation sequencing within the last 5 years in all fields of medicine, WES has become a valuable technique for screening all the protein coding regions, which constitutes ~1% of the human genome. The application of whole exome sequencing in genetic diagnostics is aimed for the discovery of novel disease-causing genes (Table 3.1) [22, 23]. WES successfully increases the yield of identification of new causative genes in genetic eye diseases [15, 24–28]. However, approximately 85% of known causative mutations occur in exonic regions that encode proteins, indicating that WES is unable to elucidate the cause of the remaining ~15% of cases. Whole genome sequencing (WGS), on the other hand, screens through the entire genome including coding regions, noncoding regions, and regulatory DNA sequences. In genetic medicine, WGS increases the yield of detecting genomic alterations related to Mendelian disorders and cancer [29]. Although WES often misses copy number variations (CNVs), large chromosomal structural variations (SVs), and deep intronic variants, WGS is much more powerful in detecting these special genomic alterations (Table 3.1) [30]. Furthermore, recent studies reported that variants located in deep intronic and microRNA regions could contribute to genetic eye diseases [31–35]. Therefore, WGS is a superior technique to WES for molecular

Table 3.1 Features of whole genome sequencing (WGS) vs. whole exome sequencing (WES) vs. targeted exome sequencing (TES)

Genetic variants	WGS	WES	TES
Exonic variants	Yes	Yes	Yes/Selected regions
microRNAs	Yes	No	No
Deep intronic variants	Yes	No	No
Variants in regulatory regions	Yes	No	No
Intergenic regions	Yes	No	No
InDels	Yes	Yes	Yes/Selected regions
Large InDels	Yes	Mostly undetected	Mostly undetected
Copy number variations	Yes	Mostly undetected	Mostly undetected
Structural variations	Yes	No	No

diagnosis and for the identification of new pathological genetic lesions. The comparisons between WGS, WES, and TES are displayed in the Table 3.1.

3.3 Procedures of Whole Genome Sequencing

3.3.1 Mapping and Variant Calling

Following the generation of raw data by an NGS platform, the process of quality control filtered the low-quality reads. Then the sequencing reads are mapped to the reference human genome. The mismatched sequences between reference genome and the patient's genome were evaluated by bioinformatics software. The common software involved in WGS analyses and their specific functions were summarized in Table 3.2, along with information on tools for quality control, alignment, SNVs/InDels, copy number variations, structural variations, de novo mutations, and annotation of both coding and noncoding regions. Mapping of sequence reads and variant calling procedures was similar to those in whole exome sequencing and targeted exome sequencing. Additional software packages were applied on analyses of copy number variations (e.g., CNVnator [36], etc.), structural variations (e.g., BreakDancer [37], etc.), de novo mutations (e.g., Random Forest, [38] etc.), and annotation of noncoding regions (FunSeq and ENCODE). Notably, software packages such as Integrative Genomics Viewer (IGV) and Circos (Fig. 3.1) were developed rapidly for visualizing data and information obtained from WGS. These software packages facilitated WGS data analyses and exhibition [39, 40].

3.4 Filtering Strategy

Each WGS sample produces an average mapping yield of ~4000,000called variants [41]. Choosing the best strategy for filtering data is a major challenge to researchers. First, we compared our data to databases of normal control populations such as the dbSNP (http://www.ncbi.nlm.nih.gov/snp/), 1000 Genomes (http://www.1000genomes.org/), ESP database (http://evs.gs.washington.edu/EVS/) and Exome Aggregation Consortium (ExAC, exac.broadinstitute.org), etc. Polymorphic variants should also be filtered out in patients with rare inherited eye diseases. Of note, although these databases are considered to be reflective of healthy controls, heterozygous mutations of autosomal recessive genes can still exist [42]. The next step involved using multiple software packages, such as PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.jcvi.org/www/SIFT_enst_submit.html) and MutationTaster (http://www.mutationtaster.org/), etc., to predict possible impact of an amino acid substitution on the structure and function of a human protein. Researchers should keep in mind that the results generated from the software are only computed predictions, rather than completely credible facts. Finally, databases such as Online

Table 3.2 Software used in the analyses of whole genome sequencing

Functions	Software
Quality control	Trim galore, NGS QC Toolkit, HTQC, NGSQC, FastQC
Alignment	BWA, Bowtie, SOAP
SNVs/InDels	GATK, SAMtools, VarScan, SOAPsnp
Copy number variations	SegSeq, CNVnator, ReadDepth, CNAseg
Structural variations	BreakDancer, LUMPY, CREST, GASV, SVDetect
De novo mutations	RandomForest, DNMFilter, PolyMutt, DeNovoGear
Annotation of coding regions	ANNOVAR, GAMES
Annotation of non-coding regions	FunSeq, ENCODE
Visualization tool	IGV, Circos

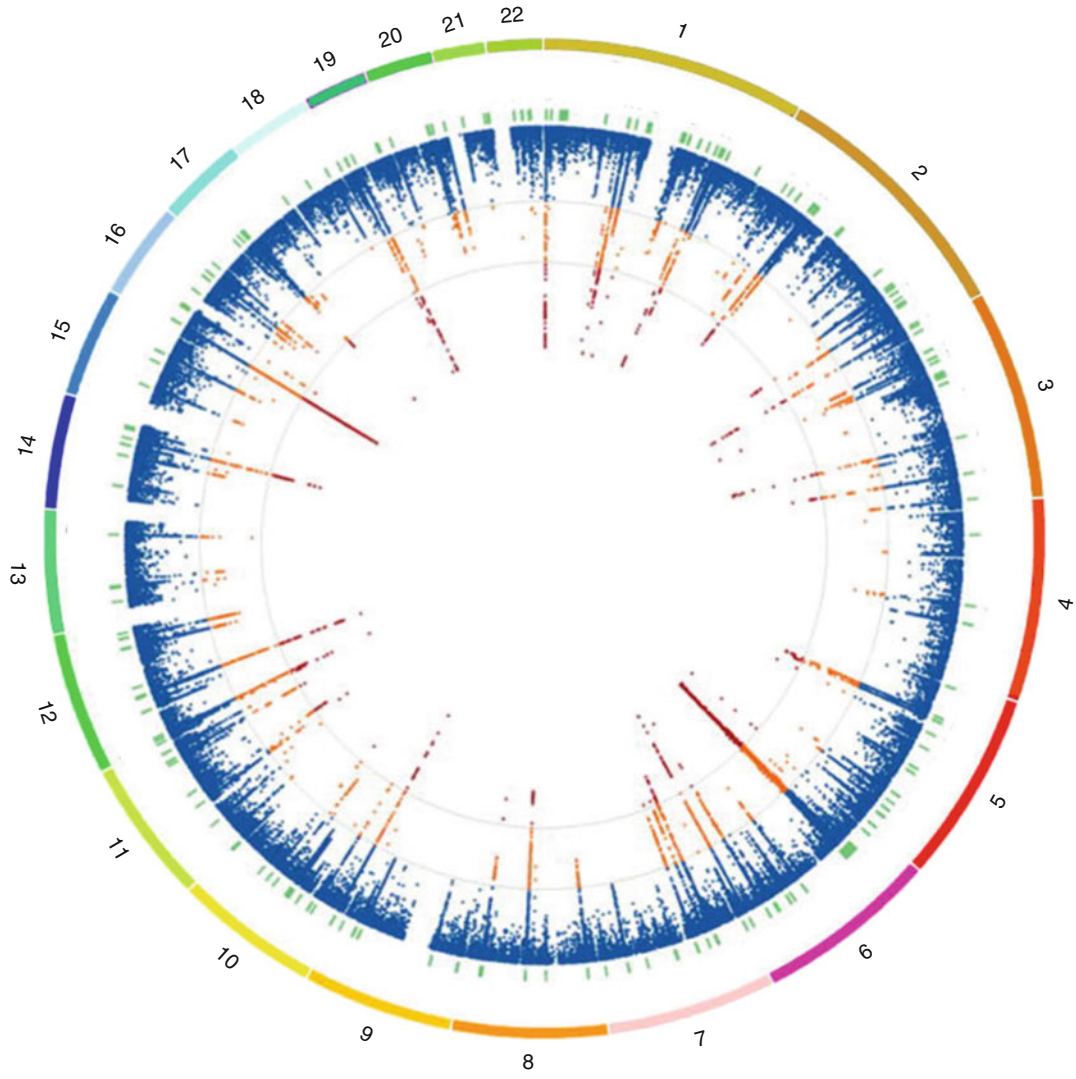

Fig. 3.1 Representation of the variants identified by whole genome sequencing using a visualized software package Circos

Mendelian Inheritance in Man (OMIM) database (http://www.omim.org/), the Human Gene Mutation Database (http://www.hgmd.org) and Catalogue of Somatic Mutations in Cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic), etc., that include information on reported gene mutations and inherited phenotype also assisted in filtering polymorphic variants. Comprehensive application of multiple databases could aid in uncovering disease-causing genes.

3.5 Applications of Whole Genome Sequencing in Genetic Eye Diseases

3.5.1 Inherited Retinal Dystrophy

Inherited retinal dystrophy (IRD) is a group of inherited eye disorders that are caused by retinal degeneration due to genetic defects, and these include retinitis pigmentosa (RP), cone-rod dystrophy (CRD), and Usher syndrome [43]. The common clinical characterization is a pathological change in rod and/or cone photoreceptor cells [44]. More than 200 causative genes associated with IRD have been identified (RetNet, https://sph.uth.edu/retnet/; RetinoGenetics, http://www.retinogenetics.org/), [45] while only approximately 50–60% cases could be attributed to specific genetic defects. Nishiguchi and colleagues recently performed whole genome sequencing on patients with autosomal recessive retinitis pigmentosa (arRP) for the first time [46]. A total of 16 unrelated patients from North America or Japan were involved in the Nishiguchi WGS study, which had an average coverage of ~66 reads. Finally, homozygous or compound heterozygous mutations in seven genes (EYS, PDE6B, USH2A, CNGB1, CERKL, RDH12, and DFNB31) associated with inherited retinal dystrophy in 8 patients were identified. Strikingly, there were two structural variations involving the noncoding parts of the genome among these known IRD genes, a ~446 kb inverted duplication in the EYS gene and a 2229-bp deletion in the USH2A gene. Additionally, a homozygous frameshift mutation in the NEK2 gene, which is previously described as the arRP gene, was revealed to be associated with arRP for the first time [46]. The homozygous frameshift in NEK2 could not have been discovered by targeted exome sequencing, and the two structural variations (a ~446 kb inverted duplication and a 2229-bp deletion) would not be detected by whole exome sequencing. Taken together, this study demonstrated the power of whole genome sequencing in detecting structural variations, especially those involving noncoding parts of the genome. WGS significantly improved the diagnostic yield on inherited retinal dystrophy. Mayer and colleagues also demonstrated the usefulness and value of whole genome sequencing [47]. They reported on a consanguineous pedigree with cone-rod dystrophy (CRD). They originally performed homozygosity mapping and combined with targeted exome sequencing and Sanger sequencing to investigate the genetic lesions in this family. However, no putative disease-causing variants were found. Subsequently, they performed whole genome sequencing on both the affected siblings, with an average read depth of 50 and 44, respectively. Interestingly, only one candidate mutation, which was located in the deep intronic regions of *PROM1* (c.2077-521A4G), was identified. In vitro splicing assay with RT-PCR and Sanger sequencing uncovered a 155-bp exon spliced between exon 18 and 19. This study is the first to report a deep intronic variant as a splicing change mutation in *PROM1*. Moreover, this mutation was not detected by combined techniques like homozygosity mapping, TES, and Sanger sequencing. Their results indicated the efficacy of WGS in detecting variants in deep intronic regions [47].

3.6 Syndromic Eye Diseases and Anophthalmia

There are several examples of applications of whole genome sequencing in diagnosing microphthalmia or syndromic eye diseases. Dr. Michael and colleagues performed clinical diagnosis using WGS on a prenatal sample

[48]. The amniotic fluid sample was from a 37-year-old pregnant woman, whose ultrasonography revealed fetal abnormalities. DNA was extracted from the amniotic fluid cells. WGS revealed a balanced translocation disrupting CHD7 at 8q12.2, a causal locus of the CHARGE syndrome. This rare syndrome was characterized as multiple organic anomalies including coloboma of the eye. This case study displayed the potential power of whole genome sequencing in prenatal genetic diagnosis [48]. Yahyavi and colleagues also used WGS to study patients with anophthalmia and hypoplasia of the optic nerve and optic chiasm and nonsense mutations in a novel gene were identified.

3.7 Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is a major cause of blindness in the elderly [49]. Both genetic and environmental factors play important roles in AMD etiology, and multiple genetic variants were reported to be associated with AMD [50]. Although genome-wide association studies (GWAS) using SNP genotyping chips are the most common methods in AMD studies, WGS has the power to discover rare SNPs, which might not be included in the existing SNP genotyping chips. Dr. Hannes and colleagues performed WGS on 2230 individuals and detected a rare SNP (minor allele frequency = 0.55%) in the C3 gene, which was found to be strongly associated with AMD [51]. With the continuous decrease in cost, WGS would be more widely adopted for the diagnosis of complex diseases such as AMD.

3.8 Retinoblastoma

Another application of whole genome sequencing is to detect somatic mutations in cancer. Retinoblastoma is a rare, pediatric cancer of the developing retina that can develop in a sporadic or a heritable form, which is initiated by the biallelic loss of RB1 [52]. Zhang et al. performed WGS on four primary human retinoblastoma samples and matched normal tissues [53]. Their results showed no genetic defects in known tumor suppressor genes or oncogenes, suggesting that the retinoblastoma genome is more stable than previously believed. Moreover, data from their WGS study also revealed somatic structural variations (SVs). Finally they demonstrated that the average number of SVs was 10 per case, ranging from 0 to 24 events per tumor sample [53].

3.9 Limitations and Challenges of WGS

Despite all the advantages of using WGS and its potentials, researchers and clinicians should also consider some remaining limitations outlined as follows:

1. The presence of false-negative mutations and false-positive results. WGS has yet to achieve a 100% coverage and accuracy with its present technological basis. Genetic counseling and correct interpretation of genetic findings still remain as challenges for both clinical and research staffs.
2. The issue of having too many candidate variants. Each WGS sample produces an average mapping yield of ~4,000,000, referred to as variants. Although filtering based on typical strategies could narrow down the number of candidates, more than 100 potential pathological variants might still need to be further analyzed. Furthermore, it is also difficult to demonstrate the pathogenicity of variants in noncoding regions. Therefore, using WGS to identify disease-causing mutations among large amounts of candidate genes remains a majority challenge.
3. The cost efficiency and data storage. Although the cost of WGS continues to decrease, it is still too high for wide application in clinical services. In addition, proper storage of the vast amount of data generated by WGS within current medical records is yet to be feasible.

3.10 Conclusions

In conclusion, WGS has revolutionized molecular genetic research and diagnostics. It is a powerful and promising technique for the identification of genetic factors that cause or contribute to genetic eye diseases. Notably, for WGS to become a more popular tool in research and routine testing for molecular diagnosis, several limitations still need to be addressed. Even though there is room for improvement, the comprehensive genetic data generated from WGS will definitely benefit clinicians and patients in molecular diagnosis by improving prognosis and indicating appropriate therapy. It is foreseeable in the near future that clinical management based on WGS data from patients with genetic eye diseases will become more personalized and more effective.

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Targeted Exome Sequencing in Japanese Patients with Retinitis Pigmentosa

4

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Abstract

Screening for causative genes in heterogeneous groups of diseases such as retinitis pigmentosa (RP) has been challenging, until recently. These days, even whole genomes can be sequenced using next-generation sequencers. This technology has enabled comprehensive screening of gene mutations in RP patients. However, whole-genome/exome sequencing is still expensive and takes considerable time and labor. In contrast, targeted exome sequencing focuses on selected genes and thereby enables faster and deeper analysis of the regions of interest at a lower cost. In this chapter, we review the data from our previous study on targeted exome sequencing in Japanese RP patients and discuss the utility and limitations of this technique. We also discuss the mutation spectrum in Japanese RP patients compared to previously reported spectra in other ethnicities.

Keywords

RP • Targeted resequencing • Exome sequencing • Japan • EYS

4.1 Introduction

Retinitis pigmentosa (RP) is the most common subtype of hereditary retinal degeneration and is both clinically and genetically heterogeneous. The prevalence is 1 in 3000–5000 individuals worldwide [1]. To date, mutations in at least 75 genes have been reported to be associated with nonsyndromic RP. In addition, more than 35 genes have been associated with syndromic RP, such as Usher syndrome and Bardet-Biedl syndrome, and other subtypes of inherited retinal diseases (RetNet; https://sph.uth.edu/retnet/). Considerable phenotypic and genetic overlap has been observed in different forms of inherited retinal diseases, which makes examinations complicated. In principle, the causative gene cannot be predicted from the phenotype. Clinical symptoms of different diseases may overlap,

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mutations in the same gene sometimes cause different phenotypes, and even individuals sharing the same mutation show a wide variety of clinical symptoms. Due to the large number of causative genes and the complexity of the phenotypes, sequence analysis of all candidate genes in a large cohort has been difficult.

Recently, the development of next-generation sequencing (NGS) technology has enabled the sequencing of all exons, or even the whole genome, of an individual, and significant progress has been made in molecular genetic studies of RP. The technology has been successfully applied to screen RP patients for pathogenic mutations in known causative genes with higher diagnosis rates (25–55%) than achieved when using conventional methods [2–8]. In addition, several novel RP genes have also been reported. Furthermore, the results have indicated that the prevalence of causative genes differs among different ethnicities. For example, the EYS gene, which is affected in ~20% of arRP cases in Japan [9, 10], is not a common cause of arRP in Northern Ireland [11]. This difference highlights the importance of specific genetic catalogues for each ethnicity.

In this article, we review the result of a targeted exome sequencing-based analysis of 193 genes in 329 Japanese patients with RP or Usher syndrome [12], the most common form of syndromic RP that is accompanied by hearing impairment. We also discuss the genetic perspective of the disease in Japanese population and pros and cons of the methodology.

4.2 Pros and Cons of Targeted Exome Sequencing

NGS technology has enabled the sequencing of hundreds of millions of regions at the same time. If the technique is used for the entire genomic DNA, every region has the same probability of being sequenced. This technique is called whole-genome sequencing (WGS), and it is useful for obtaining an entire overview of the genome. However, the dataset contains an enormous amount of information, which is not necessary for many specific purposes. In contrast to whole-genome sequencing, whole-exome sequencing (WES) reduces the cost, time, and the volume of the data by reading only the exons, but not the introns. In addition to WGS/WES, there is another NGS method called targeted exome sequencing. Targeted exome sequencing reads only the targeted regions of the genome. Any regions of interest, including exons and introns, can be enriched and sequenced. The technique can be applied to screen a list of genes that are known to be associated with diseases of interest.

While WES or WGS may become a standard screening method in the near future, targeted exome resequencing still has a number of advantages. Firstly, targeted exome resequencing can achieve a higher coverage rate in the region of interest. Because the data output quantity by NGS depends on the total lengths of the regions and the read depth, targeted exome sequencing can increase the read depth while reducing the total length of regions. Secondly, it allows more patients to be screened in a single assay. While WES reads a myriad of regions in a smaller number of patients, targeted exome resequencing analyzes a limited number of regions in more patients. These features lead to faster and less expensive examinations per patient [13]. Other advantages include smaller datasets, which makes bioinformatics analysis faster and easier. In addition, targeted exome resequencing can reduce the risk of incidentally finding specific variants, which can lead to ethical problems.

The major drawback of targeted exome sequencing is the selection of the analyzed region itself. If researchers want to analyze a new gene that is not included in previous sequencing analyses, they have to sequence the gene from the beginning while WGS/WES contains the information of all genes. If either WGS or WES has been performed previously, the researchers can start with the pre-existing data. The second drawback is a cost for the custom design. Whereas ready-made kits containing fixed primer sets can be used in WES, targeted exome sequencing requires order-made kits for generating libraries. Although targeted exome sequencing is still less expensive than WES

despite the cost of the custom design, this advantage will be lost if sequencing costs continue to decline.

As stated above, the advantages of targeted exome sequencing include reduced cost and the ability to handle many samples. This technique is well suited for screening multiple, previously identified genes and can be used to efficiently identify patients with mutations in those genes. On the other hand, WES is practical for detecting new causative genes.

4.3 Laboratory

4.3.1 Principal of Targeted Exome Sequencing

Genomic DNA extracted from the peripheral blood is ideal for this method. Genomic DNA also can be extracted from other samples, such as saliva, but extraction from other samples often results in smaller amounts and less pure DNA.

After genomic DNA extraction, the region of interest should be enriched. Amplicon-based library preparation offers a powerful option for selected enrichment, and at least three approaches are currently being used for the preparation and enrichment of regions of interest [14]. Commercially available kits for custom design and selected enrichment currently available from four major companies are listed in Table 4.1.

One method for preparation and enrichment involves a multiplex PCR-based library preparation. Ampliseq Technology from Life Technologies, Inc. is one example that uses this strategy. Ion Ampliseq uses an ultrahigh multiplex PCR technology to generate thousands of amplicons. Genomic DNA and thousands of primer pairs are pooled together in a single tube, and the regions of interest are selectively amplified using multiplex PCR. Another method is single-plex PCR-based target enrichment, which utilizes microdroplet PCR. In this technology, picoliter-sized droplets work as individual

Table 4.1 List of library preparation kits for targeted sequencing

Kits	Input DNA	Duration time[a]	Design software	Target size
Agilent				
SureSelectXT Custom	200 ng or 3 μg	1~2.25 days	SureDesign	1 kb~24 Mb
HaloPlex Custom Kit	225 ng	>6 h	SureDesign	1 kb~5 Mb
Roche NimbleGen				
SeqCap EZ Choice Library/	100 ng (for Illumina)	1.25 days (for Illumina)	NimbleDesign	7 Mb/
SeqCap EZ Choice XL Library	500 ng (for Roche454)	3.5 days (for Roche454)		200 Mb
Illumina				
Nextera Rapid Capture Custom Enrichment	50 ng	1.5 days		37 Mb
TrueSight Sequencing Panels	50 ng	1.5 days		12 Mb (4813 genes)
TruSeq Custom Amplicon	50 ng	2 days	DesignStudio	650 kb (1536 amplicons)
Lifetechnologies				
Ion AmpliSeq Custom DNA Panels	10 ng	>3.5 h	Ion Ampliseq Designer	1 kb~5 Mb
Ion TargetSeq Custom Enrichment Kit	1 μg	1.75~3.25 days	Not available[b]	100 kb~10 Mb
TargetSeq Custom Enrichment Kit	3 μg	1.75~3.25 days	Not available[b]	100 kb~60 Mb

[a]Time required to enrich target regions
[b]Send the list of target regions to Lifetechnologies via email

reaction vessels, allowing over one million unique PCR reactions per sample. Genomic DNA template mixture and primer pairs that cover the regions of interest are made into droplets and merged on the microfluidic chip. Microdroplet PCR technology can greatly reduce the amount of reagents required and is suitable for parallel amplification in a large number of samples [15]. In contrast, there is a group of methods using a different approach: these methods capture the target regions and then enrich the regions using multiplex PCR. HaloPlex technology from Agilent Technologies and TruSeq from Illumina are included in this group. The method uses pools of oligonucleotides or probes that are designed to hybridize with targeted regions and that are bound to magnetic beads. After binding to genomic DNA, these selectively hybridized sequences are pulled down with magnets and unbound probes are washed away. Finally, the targeted regions are amplified, providing amplicon product ready for selective sequencing.

It should be noted that WES also includes this step. If probes are used that are designed to bind to all exons, the sample can be used for WES. Thus, WES can be considered a form of targeted exome sequencing that targets all exons. While several ready-made kits using different techniques are currently available for exome sequencing, targeted exome requires custom design. Nonetheless, most companies provide online custom design tools, and the process is usually not difficult. It is important to carefully select the regions of interest because they cannot be changed after the libraries are made.

4.3.2 Data Analysis

Although the volume of the datasets obtained from targeted exome sequencing is smaller than the volume obtained from WGS/WES, biostatistical analysis is required to interpret the results. In brief, at first, millions to billions of reads are mapped to a best-fit location on the reference sequence. After the alignment, variants are called. In order to improve the accuracy of the data, it is recommended to perform a local realignment, removal of PCR duplicates, and recalibration of variant quality scores. It is also recommended to filter variants based on quality criteria in order to reduce false-positive calls.

Common variants should be excluded from further analysis, especially when dealing with a rare disease like RP. Several public databases, such as dbSNP and 1000 Genomes, as well as in-house databases, are useful for this purpose. This step usually substantially reduces the number of potential candidate mutations [16].

The most important, but difficult, process is determination of the pathogenicity of the variants. This process widely differs depending on the strategy that the investigator uses [17]. Because many disease-causing mutations are found in coding regions and in flanking regions, one common approach is to limit analyses to variants in those regions, whether starting with genome or exome data set. To narrow down the number of candidate variants, statistical prediction tools are often applied. There are several well-established in silico prediction programs that determine whether the variants have a functional effect and occur in conserved gene regions. However, it should be noted that the predictions are not always correct. The prediction programs might misidentify pathogenic mutations as benign or include benign variants as pathogenic. Employing several tools might increase the specificity but may also decrease sensitivity. The results should be confirmed by segregation analysis whenever possible, i.e., available family member should be screened for the mutation, and the genotype should be checked for compatibility with the phenotype.

The identified mutations should be confirmed with direct sequencing. Although NGS provides reliable data because of the improved technology and bioinformatics, the data from regions with low read depth, or from highly repetitive or GC rich regions, can be unstable.

4.3.3 Example of Practice

Here, we provide an example of practice according to our previous study [12].

4.3.3.1 Data Acquisition

Preparation of Genomic DNA Genomic DNA was extracted from peripheral blood using a DNA extraction kit (QuickGene-610 L; Fujifilm, Minato, Tokyo, Japan). The quantity and quality of the DNA was verified with a dsDNA HS Assay kit using a Qubit® (Life Technologies, Carlsbad, CA, USA) and a NanoDrop® spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Custom Design of Target Regions SureDesign software (Agilent Technologies) was used to design the custom HaloPlex capture assay. The capture panel was designed to cover all coding and noncoding exons with flanking exon/intron boundaries (± 25 bp) of the 193 retinal and optical disease genes that were reported in RetNet at the time of the study. In addition, to identify new causative genes, 172 genes expressed at the highest level in rod or cone photoreceptors were also included. The capture panel consisted of 2,433,298 base pairs (bp) and covered 365 genes in total.

Target Capture A library of genomic DNA fragments was created using the custom design HaloPlex Target Enrichment 2.5 Mb kit (Agilent Technologies, Santa Clara, CA, USA). Briefly, the protocol consisted of the following five steps: (1) Genomic DNA (225 ng) samples were fragmented with eight different restriction enzymes and denatured. (2) Probes specifically designed to capture the region of interest were added and hybridized to the target fragments. During hybridization, the fragments were circularized and sequencing motifs, including index sequences, were incorporated. (3) The target DNA fragments were retrieved with magnetic streptavidin beads, and, subsequently, only perfectly hybridized fragments were closed by ligation. (4) Circular DNA targets were amplified and products were purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA). (5) The resultant libraries were evaluated using the Agilent 2200 TapeStation and libraries containing high amounts of adaptor-dimer were purified again using AMPureXP beads. For each capture reaction, 47 or 48 libraries were pooled. The concentration of the pooled libraries was determined using the 2100 Bioanalyser (Agilent Technologies).

Sequencing The pooled libraries were quantified and sequenced on the Illumina HiSeq 2500 as 100-bp paired-end reads as instructed by the manufacturer.

4.3.3.2 Data Analysis

Processing of Raw Data Sequence reads were mapped and aligned to the reference human genome (NCBI Build 37) using the Burrows-Wheeler Aligner [18]. The adapter sequence (AGATCG) was trimmed using custom Perl script. Base quality values were recalibrated and local realignment was performed. The Genome Analysis Toolkit [19] was used for single nucleotide variant and insertion/deletion (indel) calling. The SNPs and indels were filtered against data from the following databases: dbSNP (Build 138) (National Centre for Biotechnology Information, http://www.ncbi.nlm.nih.gov/SNP/), 1000 Genomes [20], NHLBI GO Exome Sequencing Project (ESP6500, http://evs.gs.washington.edu/EVS/), and the Human Genetic Variation Database (HGVD, http://www.genome.med.kyoto-u.ac.jp/SnpDB/). HGVD contains the exome sequencing data of 1208 Japanese control subjects. Variant annotation was performed using ANNOVAR [21].

Filtration and Prioritization of Variants Variants that had an allele frequency > 0.5% (for recessive variants) or > 0.1% (for dominant variants) in 1000 Genomes, ESP6500, or HGVD were excluded based on the prevalence of RP. Then, mutations listed in the

Human Gene Mutation Database (HGMD) or those identified as pathogenic alterations in previous publications were extracted and considered pathogenic. Nonsense and frameshift variants in coding sequences were also considered pathogenic.

In Silico Prediction of Variants Five computational prediction tools for amino acid substitutions (SIFT, Polyphen2, LRT, MutationTaster, and MutationAssessor) and two computational tools for estimating evolutionary conservation (PhyloP and GERP++) obtained from dbNSFP [22] were used to assess the pathogenicity of novel missense variants. A variant was classified as pathogenic if the following criteria applied: predicted to be pathogenic by at least three of five missense prediction programs (SIFT, Polyphen2, LRT, MutationTaster, and MutationAssessor) and evolutionary conservation scores was > 0 in both PhyloP and GERP++. In addition, for splice-site variants, the prediction program MaxEntScan was used, and the variants were considered pathogenic if the score differed > 5 between the wild-type and mutated sequences.

Validation of Variants Variants were adopted as disease-causing mutations only when that matched the patients' phenotype and the reported inheritance pattern of the respective genes. All mutations and potential pathogenic variants were confirmed by using conventional Sanger sequencing. An Applied Biosystems (ABI) 3130xl Genetic Analyser (Life Technologies) was used for Sanger sequencing. Segregation analysis was performed if DNA from family members was available. If a novel pathogenic variant in the dominant genes was detected in simplex case, it was considered to be a disease-causing mutation only if it was confirmed by parental testing to have occurred de novo.

Results Three hundred and twenty-nine patients were recruited for this study, and a total of 103 distinct pathogenic mutations were identified for 115/317 (36.3%) RP patients and 6/12 (50%) Usher syndrome patients. The percentage of causative genes in this cohort is shown in Fig. 4.1.

4.4 Epidemiology

To date, numerous studies using different methods for the molecular diagnosis of RP have been conducted, including Sanger sequencing, arrayed primer extension chips, and NGS technology. The results have indicated that the prevalence of causative genes and the spectrum of mutations can differ significantly among different ethnicities.

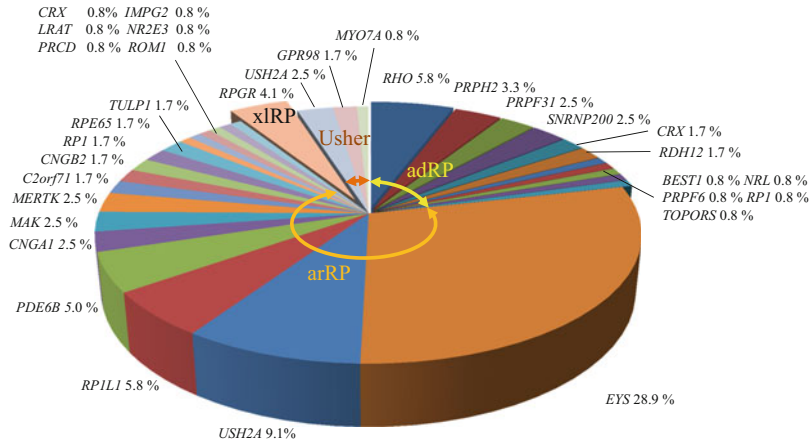

Fig. 4.1 The percentage of causative genes with each inheritance trait that were identified in 115 RP patients and six Usher syndrome patients. Mutations in *EYS* and *USH2A* were common in the population (Adapted from Ref. [12])

Our group conducted a comprehensive genetic screening of 317 Japanese RP patients and 12 Japanese Usher syndrome patients using targeted resequencing. We demonstrated that the prevalence of causative genes differed in Japanese populations when compared to the causative genes in other ethnicities [12]. Our data confirmed the high prevalence of mutations in *EYS*: *EYS* is the causative gene in 15% of Japanese arRP patients and in up to 11% (35/317) of the Japanese RP population in the all inheritance, which is the highest percentage by which a single gene accounts for RP [23]. The association between *EYS* mutations and RP was first reported in 2008 [24]. Subsequently, several reports demonstrated a prevalence of *EYS* mutations in 0% of Northern Ireland RP patients [11], 3–7% of Chinese arRP patients [8, 25], and 5–10% of European arRP patients [7, 26, 27], whereas, the prevalence of *EYS*-associated RP was as high as 20% in Japanese arRP patients [9, 10]. Notably, the allele frequency of mutations in this gene also varies significantly among ethnicities. The most frequent *EYS* mutations in Japanese RP patients, c.8805C>A and c.4957dupA, are rare or not detected in European RP patients or in other Asian RP patients [8, 25, 28, 29].

On the other hand, *USH2A*, which is the most frequently affected gene in arRP patients in China, North America, and Europe, was the second most frequently affected gene in our Japanese cohort. The mutation frequency of *USH2A* in arRP patients was 10–12% in China [8, 25], 7–23% in North America [30, 31], and 7–14% in Europe [32, 33], whereas the mutation frequency was 4–5% in Japan [12, 34]. The mutations frequently observed in Caucasian RP patients, such as *USH2A* p. C759F, were not observed in either Japanese or Chinese cohorts [8, 25, 34]. These data underscore the different mutation spectrum of *USH2A* between Caucasian patients and Asian patients. However, the two large genes, *EYS* and *USH2A*, are considered to be one of the most affected genes in many ethnicities worldwide.

As for causative genes in adRP patients, RHO, PRPH2, and PRPF31 was the top three in our Japanese cohort, which is consistent with the report of the top genes in a North American cohort [35]. However, the prevalence of mutations in RHO was 9% in Japanese adRP patients, which was lower than the prevalence in Chinese (21%) [8], Caucasian (16–29%) [35–38], and Mexican (17%) [39] patients.

As stated above, the prevalence of causative genes, as well as the frequency of each mutation, differs between ethnicities. Therefore, mutation screening and characterization of the mutation spectrum in each ethnicity will improve our understanding of the entire genetic background and will provide comprehensive knowledge of genes affecting inherited retinal degeneration. Recent developments in NGS technology provide powerful new approaches that enable efficient and cost-effective molecular testing of entire genomes. Further studies using this technology will expand our knowledge of inherited retinal degenerations.

4.5 Compliance with Ethical Requirements

Authors Maho Oishi, Akio Oishi, and Nagahisa Yoshimura declare that they have no conflict of interest.

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

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Genetic Epidemiology of Congenital Cataracts and Autosomal Recessive Retinal Degenerations in Pakistan

5

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Abstract

The National Eye Institute (NEI) of the United States, the Centre for Excellence in Molecular Biology (CEMB), and Allama Iqbal Medical College in Lahore, Pakistan collaborate in an ongoing international genetic collaboration to identify the genes and mutations responsible for retinitis pigmentosa and congenital cataracts in the Pakistani population. To date, we have recruited a total of 115 consanguineous families with autosomal recessive congenital cataract (arCC) and 138 consanguineous families with autosomal recessive retinal dystrophy (arRD). After undergoing linkage analysis and sequencing of candidate genes in identified linked regions, unmapped families were subjected to homozygosity exclusion mapping by screening closely flanking microsatellite markers at known candidate gene/loci, followed by Sanger sequencing of the nearby candidate gene if homozygosity was present. Analysis of these families has resulted in identification of disease loci or genes causing disease in 42 families with arCC and 96 families with (RD), including novel disease-causing genes in 5 of these families. Since selection of both families and candidate loci was unbiased, this approach allowed an

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accurate estimate of the contributions of various genes to arCC and arRD in Pakistan. The causative genes remain unidentified in about 2/3 arCC families and 1/3 of arRD families in our study, making next-generation sequencing an ideal method for identification of new genes.

Keywords
Homozygosity mapping • Genetic analysis • Retinal dystrophies • Congenital cataracts • Pakistan

5.1 Pakistani Population Characteristics

The Pakistan population has been a rich source for identifying genes causing or contributing to autosomal recessive diseases. There are several reasons for that. First, consanguineous marriages are very common in Pakistan. About 50–60% of all marriages in Pakistan are reported to be consanguineous due to sociocultural and religious reasons [8]. It has been well known for 60 years that consanguinity often poses an increased probability of recessive disorders caused by homozygous mutations inherited from inbreeding parents (IBD, identical by descent). Second, the characteristics of low mobility and isolated villages in Pakistan further emphasize autosomal recessive diseases in this region, and families can often be collected as units. Third, families in Pakistan tend to be large, so that the chances of mapping disease loci using linkage analysis are greatly improved.

5.2 Collaboration Between the NEI, the CEMB, and AIMC

The collaboration between the NEI and the Centre for Excellence in Molecular Biology (CEMB) was initiated in 2004, aiming to identify the genetic causes of congenital cataracts (CC) and autosomal recessive retinal dystrophies (arRD) in the Pakistani population. NEI annually ascertain 25CC and 25 arRD families mostly from the Punjab region of Pakistan. It is the most populous of the four provinces of Pakistan, with approximately 56% of the country's total population. The CEMB is a large research center upgraded from the University Centre of the Punjab for advanced studies in molecular biology in 1986. Allama Iqbal Medical College, one of the top medical universities in Pakistan, joined the collaboration in 2010. From 2004 to 2012, the collaboration has been extremely productive, yielding identification of disease loci or genes in 32 arCC families and 69 arRD families, including novel genes identifying new biological pathways in the lens and retina, including CRYBB3, FYCO1, and EPHA2 for congenital cataracts, ZNF513 for retinitis pigmentosa, and SLC24A1 for congenital stationary night blindness (CSNB). Given that Pakistan has the highest prevalence of consanguineous marriages in the world [4], we also applied homozygosity mapping followed by mutation screening in 83 additional consanguineous families with arCC and 69 consanguineous families with arRD, which enabled an estimate of the contributions of various known candidate genes to arCC and arRD in Pakistan.

5.3 Approaches to Identifying Genes and Loci

5.3.1 Linkage Analysis

A total of 382 highly polymorphic microsatellite markers (Applied Biosystems PRISM Linkage Mapping Set MD-10, Foster City, CA) were used for linkage analysis. PCR products were separated on an ABI 3130 DNA Analyzer, and alleles were assigned with Gene Mapper Software version 4.0 (Applied Biosystems).

Two-point linkage analyses were performed with the FASTLINK version of MLINK from the LINKAGE Program Package. Both diseases were analyzed as a fully penetrant trait with a disease allele frequency of 0.001, and equal allele frequencies were arbitrarily used for all markers in the genome-wide scan. Marker allele frequencies were calculated from 100 unrelated Pakistani control individuals for fine mapping. The order of the marker and the distances between the markers were obtained from the Genethon (http://www.broadinstitute.org/cgi-bin/contig/phys_map) or Marshfield (http://research.marshfieldclinic.org/genetics/GeneticResearch/compMaps.asp) databases.

5.3.2 Homozygosity Mapping

In this study, 33 candidate genes and loci were selected for arCC screening (Table 5.1) based on the Cat-Map database (http://cat-map.wustl.edu/), and 181 candidate genes and loci were selected for arRD screening based on the RetNet database (https://sph.uth.edu/Retnet/, Table 5.2). The selected genes are commonly involved in CC,

Table 5.1 Summary of genes tested for homozygosity in arCC families

No.	Gene or locus	Inheritance	Chromosomal location	Region in Mbp
1	*EPHA2*	AD,AR	1p36.1	16.45–16.48
2	*FOXE3*	AD,AR	1p32	47.88
3	*GJA8*	AD,AR	1q21–q25	147.37–147.38
4	*CRYGC*	AD	2q33–q35	208.99
5	*CRYGD*	AD	2q33–q35	208.98
6	*FYCO1*	AD	3p21.31	45.95–46.03
7	*BFSP2*	AD,AR	3q21–q22	133.11–133.19
8	*CRYGS*	AD	3q26.3–qter	186.25–186.26
9	*SIL1*	AR	5q31	138.28–138.53
10	*GCNT2*	AR	6p24–p23	10.52–10.62
11	*EYA1*	AD	8q13.3	72.10–72.26
12	*GALT*	AR	9p13	34.64–34.65
13	*TDRD7*	AR	9q22.33	100.17–100.25
14	*VIM*	AD	10p13	17.27
15	*SLC16A12*	AD	10q23.13	91.19–91.29
16	*PITX3*	AD	10q25	103.98–104.00
17	*CRYAB*	AD,AR	11q23.3–q24.2	111.77–111.78
18	*MIP*	AD,AR	12q12–q14.1	56.84–56.86
19	*GJA3*	AD	13q11–13	20.71–20.73
20	*TMEM114*	AD	16p13.2	8.61–8.62
21	*HSF4*	AD,AR	16q22.1	67.19–67.20
22	*MAF*	AD	16q22–q23	79.62–79.63
23	*CRYBA1*	AD	17q11–q12	27.57–27.58
24	*GALK1*	AR	17q24	73.75–73.76
25	*FTL*	AD	19q13.33	49.46–49.47
26	*LIM2*	AR	19q13.4	51.88–51.89
27	*BFSP1*	AD,AR	20p11.23–p12.1	17.47–17.51
28	*CHMP4B*	AD	20q11.22	32.39–32.44
29	*CRYAA*	AD,AR	21q22.3	44.58–44.59
30	*CRYBB2*	AD	22q11.2	25.61–25.62
31	*CRYBB3*	AD,AR	22q11.2	25.59–25.60
32	*CRYBB1*	AD,AR	22q11.2	26.99–27.01
33	*CRYBA4*	AD	22q11.2	27.01–27.02

Table 5.2 Summary of genes tested for homozygosity in arRD families

No.	Gene or locus	Inheritance	Diseases[a]	Chromosomal location	Region in Mbp
1	*RPE65*	AR/AD	LCA, RP	1p31.2	68.89–68.92
2	*CRB1*	AR/AD	LCA, RP	1q31.3	197.17–197.45
3	*KCNJ13*	AR/AD	LCA, SVD	2q37.1	233.63–233.64
4	*RHO*	AR/AD	RP, CSNB	3q21–q24	129.24–129.25
5	*PROM1*	AR/AD	RP, SMD, CRD	4p15.32	15.96–16.08
6	*PDE6B*	AR/AD	RP, CSNB	4p16.3	0.61–0.66
7	*RP1*	AR/AD	RP	8q12.1	55.52–55.54
8	*RGR*	AR/AD	RP	10q23	86.00–86.02
9	*BEST1*	AR/AD	RP, MD, ARB	11q12.3	61.71–61.73
10	*LRP5*	AR/AD	FEVR	11q13.2	68.08–68.22
11	*NRL*	AR/AD	RP	14q11.1–q11.2	24.54–24.55
12	*RDH12*	AR/AD	LCA, RP	14q24.1	68.17–68.20
13	*NR2E3*	AR/AD	ESC, RP	15q22.32	72.10–72.11
14	*GUCY2D*	AR/AD	LCA, CRD	17p13.1	7.90–7.92
15	*AIPL1*	AR/AD	LCA, CRD	17p13.2	6.32–6.34
16	*CRX*	AR/AD	CRD, LCA, RP	19q13.32	48.32–48.35
17	*GNAT2*	AR	Achromatopsia	1p13.1	110.14–110.16
18	*RP32*	AR	RP	1p21.2–p13.3	N/A
19	*ABCA4*	AR	SMD, RP, CRD, fundus flavimaculatus	1p22.1	94.45–94.59
20	*DHDDS*	AR	RP	1p36.11	26.76–26.80
21	*RD3*	AR	LCA	1q32.3	211.64–211.67
22	*USH2A*	AR	US, RP	1q41	215.80–216.60
23	*FAM161A*	AR	RP	2p15	62.05–62.08
24	*C2orf71*	AR	RP	2p23.2	29.28–29.30
25	*ZNF513*	AR	RP	2p23.3	27.60–27.60
26	*CNGA3*	AR	Achromatopsia, CRD	2q11.2	98.96–99.02
27	*CNNM4*	AR	CRD	2q11.2	97.43–97.48
28	*MERTK*	AR	RP, CRD	2q14.1	112.66–112.79
29	*CERKL*	AR	RP, CRD	2q31.3	182.40–182.52
30	*SAG*	AR	RP, Oguchi disease	2q37.1	234.21–234.26
31	*IMPG2*	AR	RP	3q12.2–q12.3	100.94–101.04
32	*IQCB1*	AR	LCA, SLSN	3q13.33	121.48–121.55
33	*CLRN1*	AR	US, RP	3q25	150.64–150.69
34	*SLC7A14*	AR	RP	3q26.2	170.46–170.59
35	*CNGA1*	AR	RP	4p12	47.93–48.01
36	*LRAT*	AR	RP, LCA	4q32.1	155.66–155.67
37	*RP29*	AR	RP	4q32–q34	N/A
38	*CYP4V2*	AR	BCD, RP	4q35.2	187.11–187.13
39	*GPR98*	AR	US	5q14.3	89.85–90.46
40	*PDE6A*	AR	RP	5q31.2–q34	149.23–149.32
41	*GRM6*	AR	CSNB	5q35.3	178.41–178.42
42	*TULP1*	AR	RP, LCA	6p21.31	35.46–35.48
43	*MAK*	AR	RP	6p24	10.76–10.84
44	*EYS*	AR	RP	6q12	64.42–66.42
45	*LCA5*	AR	LCA	6q14.1	80.19–80.25
46	*ADAM9*	AR	CRD	8p11.22	38.85–38.96
47	*CNGB3*	AR	Achromatopsia, CD	8q21.3	87.59–87.76
48	*KCNV2*	AR	CD	9p24.2	2.72–2.73
49	*DFNB31*	AR	US	9q32	117.16–117.27

(continued)

Table 5.2 (continued)

No.	Gene or locus	Inheritance	Diseases[a]	Chromosomal location	Region in Mbp
50	*RBP3*	AR	RP	10q11.2	48.38–48.39
51	*RNANC*	AR	Congenital retinal nonattachment	10q21	N/A
52	*PCDH15*	AR	US	10q21.1	55.56–56.56
53	*CDHR1*	AR	CRD	10q23.1	85.95–85.98
54	*CDH23*	AR	US	10q23.1	73.16–73.58
55	*PDE6C*	AR	CD, achromatopsia	10q23.33	95.37–95.43
56	*RBP4*	AR	RPE degeneration	10q23.33	95.35–95.36
57	*OAT*	AR	Gyrate atrophy	10q26.13	126.08–126.11
58	*USH1C*	AR	US	11p15.1	17.51–17.57
59	*CABP4*	AR	CSNB, LCA	11q13.2	67.22–67.23
60	*MYO7A*	AR	US	11q13.5	76.84–76.93
61	*MFRP*	AR	Nanophthalmos, microphthalmos	11q23.3	119.21–119.22
62	*CACNA2D4*	AR	CD	12p13.33	1.90–2.03
63	*RDH5*	AR	Fundus albipunctatus, CD	12q13–q14	56.11–56.12
64	*CEP290*	AR	SLSN, LCA	12q21.32	88.44–88.54
65	*GRK1*	AR	CSNB	13q34	114.32–114.33
66	*RPGRIP1*	AR	LCA, CRD	14q11.2	21.75–21.82
67	*SPATA7*	AR	LCA	14q31.3	88.85–88.90
68	*TTC8*	AR	RP, BBS	14q31.3	89.29–89.34
69	*TRPM1*	AR	CSNB	15q13.3	31.29–31.45
70	*SLC24A1*	AR	CSNB	15q22.31	65.91–65.95
71	*RLBP1*	AR	RP, CRD	15q26.1	89.75–89.76
72	*RP22*	AR	RP	16p12.1–p12.3	N/A
73	*ARL2BP*	AR	RP	16q13	57.27–57.29
74	*CNGB1*	AR	RP	16q13	57.92–58.01
75	*CDH3*	AR	MD	16q22.1	68.68–68.73
76	*RGS9*	AR	Delayed cone adaptation	17q24.1	63.13–63.22
77	*PDE6G*	AR	RP	17q25	79.61–79.62
78	*USH1G*	AR	US	17q25.1	72.91–72.92
79	*PRCD*	AR	RP	17q25.1	74.52–74.54
80	*RAX2*	AR	CRD, MD	19p13.3	3.77–3.80
81	*RGS9BP*	AR	Delayed cone adaptation	19q13.12	33.16–33.17
82	*IDH3B*	AR	RP	20p13	2.63–2.64
83	*VRD1*	AR	Vitreoretinal dystrophy	22q13	N/A
84	*EMC1*	AD	RP	1p36.13	19.54–19.58
85	*NMNAT1*	AD	LCA	1p36.22	10.00–10.04
86	*MFN2*	AD	OA	1p36.22	12.04–12.07
87	*PLA2G5*	AD	Benign fleck retina	1p36–p34	20.39–20.42
88	*PRPF3*	AD	RP	1q21.1	150.29–150.33
89	*SEMA4A*	AD	RP, CRD	1q22	156.12–156.15
90	*CFH*	AD	MD	1q32	196.62–196.72
91	*NEK2*	AD	RP	1q32.2–q41	211.83–211.85
92	*SDCCAG8*	AD	BBS	1q43	243.42–243.66
93	*EFEMP1*	AD	MD	2p16	56.09–56.15
94	*SNRNP200*	AD	RP	2q11.2	96.94–96.97
95	*NPHP1*	AD	SLSN, BBS	2q13	110.88–110.96
96	*BBS5*	AD	BBS	2q31.1	170.34–170.36
97	*GNAT1*	AD	CSNB	3p21	50.23–50.24
98	*LZTFL1*	AD	BBS	3p21.3	45.86–45.88

(continued)

Table 5.2 (continued)

No.	Gene or locus	Inheritance	Diseases[a]	Chromosomal location	Region in Mbp
99	*ARL6*	AD	BBS	3q11.2	97.48–97.52
100	*RHO*	AD	RP, CSNB	3q21–q24	129.25–129.25
101	*OPA1*	AD	OA	3q28–q29	193.31–193.42
102	*DTHD1*	AD	LCA	4p14	36.28–36.35
103	*GPR125*	AD	RP	4p15.2	22.39–22.52
104	*RAB28*	AD	CRD	4p15.33	13.34–13.49
105	*LRIT3*	AD	CSNB	4q25	110.77–110.79
106	*BBS12*	AD	BBS	4q27	123.65–123.67
107	*BBS7*	AD	BBS	4q27	122.75–122.79
108	*NR2F1*	AD	OA	5q14	92.92–92.93
109	*HARS*	AD	US	5q31.3	140.05–140.07
110	*GUCA1A*	AD	CD, CRD	6p21.1	42.12–42.15
111	*GUCA1B*	AD	RP, MD	6p21.1	42.15–42.16
112	*PRPH2*	AD	RP, MD, CRD, LCA	6p21.2–p12.3	42.67–42.69
113	*RIMS1*	AD	CRD	6q12–q13	72.60–73.11
114	*ELOVL4*	AD	MD	6q14	80.62–80.66
115	*BCAMD*	AD	MD	6p12.3–q16	N/A
116	*IMPG1*	AD	MD	6q14.2–q15	76.63–76.78
117	*MCDR1*	AD	MD	6q14–q16.2	N/A
118	*RP63*	AD	RP	6q23	N/A
119	*BBS9*	AD	BBS	7p14	33.20–33.65
120	*RP9*	AD	RP	7p14.3	33.13–33.15
121	*KLHL7*	AD	RP	7p15.3	23.15–23.22
122	*MDDC (CYMD)*	AD	MD	7p21–p15	N/A
123	*TSPAN12*	AD	FEVR	7q31.31	120.43–120.50
124	*IMPDH1*	AD	RP, LCA	7q31.3–q32	128.03–128.05
125	*OPN1SW*	AD	Tritanopia	7q31.3–q32	128.41–128.42
126	*KIAA1549*	AD	RP	7q34	138.52–138.67
127	*RP1L1*	AD	RP1L1	8p23	10.46–10.51
128	*OPA6*	AD	MD, RP	8q21–q22	N/A
129	*C8orf37*	AD	CRD, RP	8q22.1	96.26–96.28
130	*GDF6*	AD	LCA, Klippel-Feil syndrome	8q22.1	97.15–97.17
131	*TOPORS*	AD	RP	9p21	32.54–32.55
132	*PRPF4*	AD	RP	9q31–q33	116.04–116.06
133	*TRIM32*	AD	BBS	9q33.1	119.45–119.46
134	*INPP5E*	AD	Joubert syndrome, MORM syndrome	9q34.3	139.32–139.33
135	*USH1K*	AD	US	10p11.21–q21.1	N/A
136	*CORD17*	AD	CRD	10q	N/A
137	*HK1*	AD	RP	10q22	71.03–71.16
138	*BBIP1*	AD	BBS	10q25.2	112.66–112.68
139	*EVR3*	AD	FEVR	11p13–p12	N/A
140	*TEAD1*	AD	Atrophia areata	11p15.2	12.70–12.97
141	*BBS1*	AD	BBS, RP	11q13.1	66.28–66.30
142	*CAPN5*	AD	Neovascular inflammatory vitreoretinopathy	11q14	76.78–76.84
143	*TMEM126A*	AD	OA	11q14.1	85.36–85.37
144	*FZD4*	AD	FEVR	11q14.2	86.66–86.67
145	*C1QTNF5*	AD	MD	11q23.3	119.21–119.22
146	*PDE6H*	AD	Achromatopsia	12p13	15.13–15.13

(continued)

Table 5.2 (continued)

No.	Gene or locus	Inheritance	Diseases[a]	Chromosomal location	Region in Mbp
147	*CODA1*	AD	Cavitary optic disc anomalies	12q13.13–q14.3	N/A
148	*BBS10*	AD	BBS	12q21.2	76.74–76.74
149	*MVK*	AD	RP	12q24	110.01–110.04
150	*C12orf65*	AD	OA	12q24.31	123.72–123.74
151	*RB1*	AD	Retinoblastoma	13q14.2	48.88–49.06
152	*ITM2B*	AD	Retinal dystrophy	13q14.3	48.81–48.84
153	*MCDR4*	AD	MD	14q11.2	N/A
154	*OTX2*	AD	LCA, microphthalmia	14q21–q22	57.27–57.28
155	*TTLL5*	AD	CD, CRD	14q24.3	76.13–76.42
156	*BBS4*	AD	BBS	15q22.3–q23	72.98–73.03
157	*USH1H*	AD	US	15q22–q23	N/A
158	*CIB2*	AD	US	15q24	78.40–78.42
159	*BBS2*	AD	BBS, RP	16q21	56.52–56.55
160	*OPA8*	AD	OA	16q21–q22.3	64.97–74.27
161	*DHX38*	AD	RP	16q22	72.13–72.15
162	*PITPNM3*	AD	CRD	17p13	6.35–6.46
163	*CACD*	AD	RP, MD, CRD, LCA	17p13	N/A
164	*PRPF8*	AD	RP	17p13.3	1.55–1.58
165	*UNC119*	AD	CRD	17q11.2	26.87–26.88
166	*GPR179*	AD	CSNB	17q21.1	36.48–36.50
167	*MKS1*	AD	BBS	17q22	56.28–56.30
168	*CA4*	AD	RP	17q23	58.23–58.24
169	*FSCN2*	AD	RP, MD	17q25	79.48–79.50
170	*OPA4*	AD	OA	18q12.2–q12.3	N/A
171	*MCDR5*	AD	MD	19q13.31–q13.32	N/A
172	*PRPF31*	AD	RP	19q13.42	54.62–54.64
173	*ABHD12*	AD	US	20p11.21	25.28–25.37
174	*KIZ*	AD	RP	20p11.23	21.11–21.23
175	*MKKS*	AD	BBS	20p12	10.39–10.41
176	*PRPF6*	AD	RP	20q13.33	62.61–62.66
177	*USH1E*	AD	US	21q21	N/A
178	*C21orf2*	AD	CRD	21q22.3	45.75–45.76
179	*OPA5*	AD	OA	22q12.1–q13.1	N/A
180	*TIMP3*	AD	Sorsby's fundus dystrophy	22q12.3	33.20–33.26
181	*IFT27*	AD	BBS	22q13.1	37.15–37.17

[a]*LCA* Leber congenital amaurosis, *RP* retinitis pigmentosa, *SVD* snowflake vitreoretinal degeneration, *CSNB* congenital stationary night blindness, *SMD* Stargardt-like macular dystrophy, *MD* macular dystrophy, *CRD* cone-rod dystrophy, *ARB* autosomal recessive bestrophinopathy, *ESC* enhanced S-cone syndrome, *US* Usher syndrome, *SLSN* Senior-Loken syndrome, *BCD* Bietti's crystalline dystrophy, *CD* cone dystrophy, *BBS* Bardet-Biedl syndrome, *OA* optic atrophy, *FEVR* familial exudative vitreoretinopathy

RD, and related disorders. Each gene was screened for homozygosity by genotyping 1 or 2 microsatellite markers (total of 51 and 202 markers for arCC and arRD, respectively). The microsatellite markers were selected based on reported high heterozygosity (0.75 or more) and were generally located within an interval of 1–2 Mb of the candidate gene. Information on the primers for amplification of microsatellite markers, marker heterozygosity, and location was obtained from the UniSTS Human Genome Database and NCBI Mapview databases. The detection of homozygosity at a given locus in consanguineous affected members was followed

by genotyping additional individuals to confirm cosegregation of markers with disease in each family. PCR products were separated on an ABI 3130 DNA Analyzer, and alleles were assigned with GeneMapper Software version 4.0 (Applied Biosystems).

5.4 Examples of Novel Genes Identified

5.4.1 Cataract

5.4.1.1 *CRYBB3*: Gene and Function

A novel locus for arCC was mapped by linkage analysis to chromosome 22 in two consanguineous Pakistani families [16]. Maximum logarithm of odds (LOD) score of 2.77 for family 60,004 and 2.04 for family 60,006 was obtained at $\theta = 0$ for marker D22S315, respectively. There was a 22.7 cM (10 Mb) linked region containing the beta-crystallin gene cluster including the genes *CRYBA4*, *CRYBB1*, *CRYBB2*, and *CRYBB3*. A homozygous missense mutation (c.493G>C, p. G165R) was detected in exon 6 of *CRYBB3* that cosegregates with the disease in both families. βB3-crystallin mRNA levels are close to those of other βγ-crystallins by real-time PCR analysis. The change in electrostatic potential was predicted to result in reducing the stability of the fourth Greek-key motif by molecular modeling, thereby reducing the stability of the entire protein [16].

5.4.1.2 *FYCO1*: Gene and Function

A genome-wide linkage analysis and fine mapping mapped a novel locus for arCC to 3p21–p22 with a summed LOD score of 33.42 for one Arab-Israeli family and 12 Pakistani families [5]. The UCSC database indicated that there were 287 genes or potential genes on the linked region. After sequencing 35 candidate genes, a homozygous missense mutation(c.1045 C>T), which results in a premature termination of translation (p. Gln349X), was identified in family 60,064 in exon 8 of the FYVE and coiled-coil domain containing 1 (*FYCO1*) gene. Eight more mutations were found after sequencing *FYCO1* in the remaining unlinked arCC families. The mutations cosegregated with the disease in each the families. *FYCO1* is a member of a PI(3)P-binding protein family which is related to the exterior of autophagosomes and mediates microtubule plus-end-directed vesicular transport. It is expressed in the mouse embryonic and adult lens and reaches its highest expressional level at P12D. The FYCO1 proteins colocalize primarily to autophagosomes and partially to microtubules and are found adjacent to Golgi. Mutations in *FYCO1* are one of the most common causes of arCC in the Pakistani population. Implication of mutations in *FYCO1* in arCC in those families suggests that autophagy is critical for lens development and transparency [5].

5.4.1.3 *EPHA2*: Gene and Function

Genome-wide linkage analysis localized a novel locus for arc on chromosome 1p in a consanguineous Pakistani family [10]. A maximum two-point LOD score of 3.56 was detected at $\theta = 0$ for marker D1S2672. The size of linked region is 10 Cm containing Eph-receptor type-A2 (*EPHA2*) that previously was considered as a causative gene for autosomal dominant CC [17, 19] and also had been shown to be associated with age-related cataract [9, 17]. Sequencing of *EPHA2* revealed a missense mutation (c.2842G>T) which leads to a glycine to tryptophan substitution (p. G948W). *EPHA2* is a member of ephrin receptor subfamily of the protein-tyrosine kinase family. EphA2 receptor interacts with ephrin-A5 in enhancing recruitment of β-catenin to N-cadherin and then regulating the adherens junction complex [6]. Bioinformatics analysis suggests that the p. G948W mutation does harm to the native structure of the protein and therefore affects its function.

5.4.2 Retinal Dystrophy

5.4.2.1 *ZNF513*: Gene and Function

A novel locus for autosomal recessive retinitis pigmentosa was mapped by linkage analysis to chromosome 2p22.3-p24.1 in a consanguineous Pakistani family (PKRP115) with a maximum two-point LOD score of 3.14, at $\theta = 0$ with

D2S165. Fine mapping identified a 12.31 cM (13.35 Mb) interval flanked by D2S220 and D2S367 that cosegregated with the disease [13]. There were 147 genes in the linked region of chromosome 2p22.3–p24.1 according to the NCBI gene browser. After sequencing 33 candidate genes, a homozygous missense mutation (c.1015T>C, p. C339R) was detected in exon 4 of the zinc finger 513 gene (*ZNF513*). The mutation was found in all affected members of the family but absent in all the unaffected individuals. In situ hybridization of human retina found that znf513 was expressed in the retina, especially in the outer nuclear layer, inner nuclear layer, and photoreceptors. Knockdown of znf513 in zebrafish embryos cause retinal thinning with specific loss of photoreceptors, which were rescued by coinjection with wild-type (WT) znf513 mRNA. In transfected COS-7 cells, both normal and p. C339R mutant ZNF513 proteins with fluorescence tag localized indistinguishably in nucleus but not in nucleolus. Chromatin immunoprecipitation (ChIP) analysis shows that only the wild-type but not the mutant ZNF513 binds to the Pax6, Sp4, Arr3, Irbp, and photoreceptor opsin promoters. These results suggest that the *ZNF513* p. C339R mutation is responsible for RP in this family and that *ZNF513* plays a key role in the regulation of photoreceptor-specific genes in retinal development and photoreceptor maintenance [12].

5.4.2.2 *SLC24A1*: Gene and Function

A genome-wide linkage scan localized the disease locus in a large multigenerational Pakistani family with congenital stationary night blindness (CSNB) to chromosome 15q. A maximum two-point LOD score of 5.92 at $\theta = 0$ was obtained with D15S993. Fine mapping markers refined the linked interval to a 10.41 cM (6.53 Mb) region between markers D15S974 and D15S1025, which harbors 109 genes according to UCSC database. Candidate genes were prioritized for sequencing according to their known function and available expression data in the retina. A 2 bp deletion (c.1613_1614del, p. F538CfsX23) in *SLC24A1*, a sodium-calcium exchanger, was identified and segregated with the disease. Expression analysis using mouse ocular tissues shows that Slc24a1 is expressed in the retina around postnatal day 7. In situ and immunohistological studies localized both SLC24A1 and Slc24a1 to the inner segment, outer and inner nuclear layers, and ganglion cells of the retina [15]. A dynamic balance between influx via cGMP-gated (CNG) channels and extrusion via Na+/Ca2+, K+ exchangers (NCKX1) was believed to control the concentration of Ca2+ in rod outer segments. The deletion of NCKX1 (Slc24a1) in mice led to malformed outer segment disks, decreased expression and function of rod CNG channels, and a subsequent 100-fold reduction in rod responses, but cone responses were preserved. Toxic Ca2+ buildup may be prevented by the compensating loss of CNG channel function in the mouse model with deleted NCKX1, which may explain the stationary nature of the CSNB in humans [18].

5.5 Mutation Spectrum of arCC and arRD in Consanguineous Pakistani Families

5.5.1 Cataract

The mutation spectrum of total 115 consanguineous Pakistani families (83 families underwent homozygosity mapping and 32 families mapped by genome-wide linkage analysis) were shown in Fig. 5.1. *FYCO1* was the gene most commonly involved with causative mutations identified in 13.0% of the families, while both *FYCO1* and *CRYBB3* together accounted for 18.2% of arCC in the families studied. In addition, the percentage of arCC cases that can be attributed to the other genes in our study cohort is about 2.6% for *EPHA2* (3/115) and *GALK1* (3/115), about 1.7% for *CRYAB* (2/115) and *SIL1* (2/115), and about 0.9% for *FOXE3* (1/115), *TDRD7* (1/115), *MIP* (1/115), *HSF4* (1/115), and *CRYBA4* (1/115). Taken together, these genes and loci account for about 36.5% of inherited cataracts in this group of families from Pakistan. For the remaining 73 (63.5%) families without causative mutations identified, there are 11 families

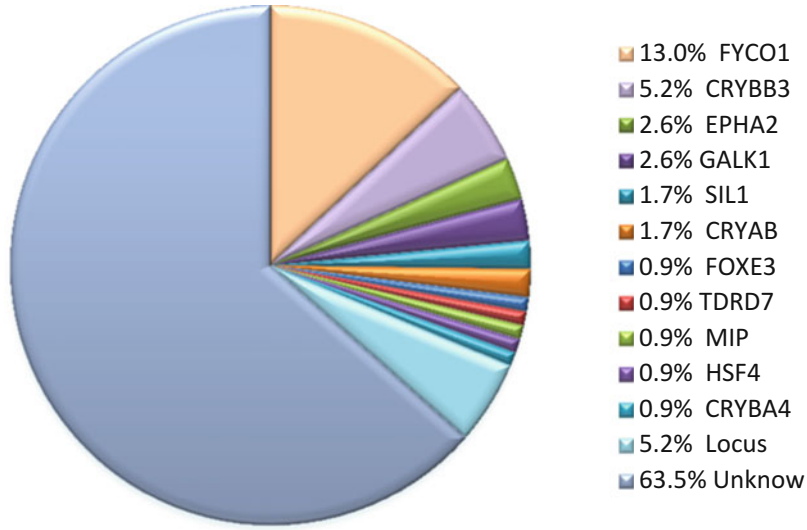


Fig. 5.1 Frequencies of genes contributing to CC in the Pakistani population

showing homozygosity in at least one of the loci which contains the known arCC genes. However, no possible causative mutation in those genes was identified.

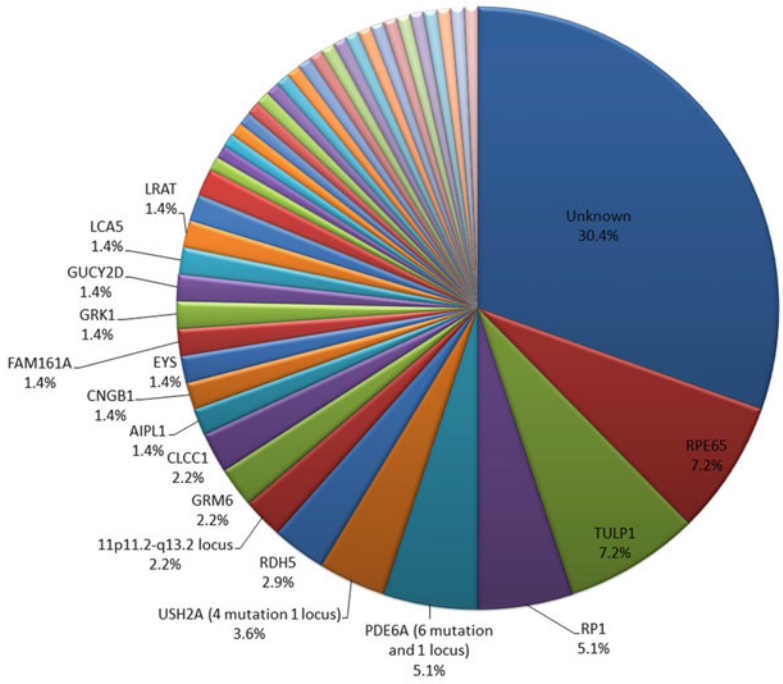
5.5.2 Autosomal Recessive Retinal Dystrophies

Combining the 69 families that underwent homozygosity mapping with the previous 69 families scanned by genome-wide linkage analysis, the mutation spectrum of total 138 consanguineous Pakistani families was shown in Fig. 5.2. *RPE65* and *TULP1* (each 7.2%) were the most frequently mutated genes in Pakistani patients, followed by *RP1* (5.1%) and *PDE6A* (5.1%). TULP1 is the most frequently mutated gene, contributing about 7.7%, followed by RP1 (5.4%) and PDE6A (5.4%). Each of the other genes accounted for less than 4% of the arRD burden. On the other hand, Pakistani population remains as a rich source for the identification of novel genes causing arRD since we didn't identify the causative mutations in 42 families (30.4%) with arRD, 31 of which were excluded by homozygosity mapping, 7 of which showed homozygosity in at least one of the 181 regions containing known arRD genes or loci but harbored no variation in the candidate genes, and 4 of which have only benign variation detected.

5.6 Discussion

The Pakistan population provides an almost unique opportunity to delineate the genetic architecture of autosomal recessive diseases, primarily because of the high level of consanguinity. Consanguinity, with its concomitant increase in the coefficient inbreeding, not only increases the frequency of recessive diseases but also increases the likelihood of homozygous mutations, which makes them easier to map. Homozygosity mapping serves as one of the most robust gene screening strategies in human genetics, is particularly suited for genetically heterogeneous conditions that are difficult to localize with other genetic techniques, and might be resistant to identification by complicated biochemical approaches. Including our previous work in this ongoing project as well as current homozygosity mapping study together, we have explored 115 consanguineous arCC families and 138 consanguineous arRD families.

Overall, there are 25 variations in 11 genes and 6 loci detected in arCC families, and these



Gene or Locus	Families	%
Total	138	100.00
Unknown	42	30.4
RPE65	10	7.2
TULP1	10	7.2
RP1	7	5.1
PDE6A (6 mutation and 1 locus)	7	5.1
USH2A (4 mutation 1 locus)	5	3.6
RDH5	4	2.9
11p11.2-q13.2 locus	3	2.2
GRM6	3	2.2
CLCC1	3	2.2
AIPL1	2	1.4
CNGB1	2	1.4
EYS	2	1.4
FAM161A	2	1.4
GRK1	2	1.4
GUCY2D	2	1.4
LCA5	2	1.4
LRAT	2	1.4
PDE6β	2	1.4
RLBP1	2	1.4
PROM1	1	0.7
CNGB3	1	0.7
BBS2	1	0.7
BBS3	1	0.7
BBS8	1	0.7
BBS12	1	0.7
CDHR1	1	0.7
CEP290	1	0.7
CERKL	1	0.7
CNGA1	1	0.7
CNGA3	1	0.7
CRB1	1	0.7
GNAT1	1	0.7
IMPDH1	1	0.7
MERTK	1	0.7
NR2E3	1	0.7
RBP3	1	0.7
RDH12	1	0.7
RPGRIP1	1	0.7
SLC24A1	1	0.7
ZNF513	1	0.7
OAT locus	1	0.7
SAG locus	1	0.7
TTPA locus	1	0.7

Fig. 5.2 Frequency of genes contributing to arRD in the Pakistani population

contribute to the disease in 36.5% of all arCC families (42/115). The frequency of involvement of the loci and genes analyzed in the Pakistan families is summarized in Fig. 5.1. *FYCO1* was the gene most commonly involved, with causative mutations identified in 13.0% of the families. *CRYBB3* and *EPHA2* accounted for 5.2% and 2.6% of arCC in the families studied, respectively. Other genes or loci were only detected in one or two families each in this cohort, contributing to less than 2% of the arCC families in Pakistan. To our knowledge, this is the first systematic description of the distribution of causative genes for arCC in the Pakistani population. Since consanguineous marriages are also very common in Saudi Arabia, their inheritance pattern is similar to that seen in Pakistan. Aldahmesh et al. found likely causative mutation in about 64.7% (11/17) of arCC Saudi Arabian families, including *CRYBB1* 41.1% (7/17), *FYCO* 15.9% (1/17), *EPHA* 25.9% (1/17), *BFSP* 25.9% (1/17), and *GCNT* 25.9% (1/17) [2]. However, the most frequent disease-causing gene in the two populations is different. *FYCO1* accounts for 13% of the diseases in Pakistani, while only explains 5.9% in Saudi Arabian. Conversely, *CRYBB1* mutations cause 41.1% of arCC in Saudi Arabian families. However, no mutations of *CRYBB1* were seen in the Pakistani arCC families in the present study. While the number of families in the Saudi study is small, the results strongly suggest that the mutation spectrum is significantly different in the two populations.

For arRD, so far we have identified variations in 39 genes and 7 loci, and these collectively account for disease in 69.6% of all families (96/138). The percentage of families that had mutated genes or identified loci are shown in Fig. 5.2, in decreasing order: *RPE65*, 7.2% (10/138); *TULP1*, 7.2% (10/138); *RP1*, 5.1% (7/138); *PDE6A* and locus, 5.1% (7/138);

USH2A, 3.6% (5/138); *RDH5*, 2.9% (4/138); 11p11.2–q13.2 loci, 2.2% (3/138); *GRM6*, 2.2% (3/138); and *CLCC1*, 2.2% (3/138). Other genes or loci were only identified in one or two families in this cohort and accounted for less than 2% of the arRD population in Pakistan. In a recent report which summarized 103 published Pakistani RD families [11], mutations were found in *AIPL1*, 11.7% (12/103); *CRB1*, 7.8% (8/103); *TULP1*, 7.8% (8/103); *RPGRIP1*, 4.9% (5/103); *RP1*, 3.9% (4/103); *SEMA4A*, 3.9% (4/103); *LCA5*, 3.9% (4/103); and *PDE6A*, 3.9% (4/103), in decreasing order, although this frequency presumable reflected mapped families only and thus were not exhaustive. In addition, a possible reason that *AIPL1* and *CRB1* were the most frequent causative genes in this study probably relates to the high frequency of LCA families, which accounted for around 20% arRD families, while in our patient cohort, there were fewer LCA cases. On the other hand, the most highly mutated genes vary remarkably among the populations of different ethnic origins. *RPE65* and *TULP1* were the genes most frequently mutated in Pakistani patients with arRD, while the genes found to be most frequently mutated in other populations were *RP1* in Saudi Arabian [1], *RDH12* in Spanish [3], and *USH2A* worldwide [7].

We failed to uncover the pathological variation in 63.5% and 30.4% of families in our arCC and arRD patient cohorts, respectively. Thus, these families are suitable for identification of new genes. Because many of these are relatively small in size, the most promising approach to discover novel genes after the systematic analysis of the consanguineous families described here is next-generation sequencing. In addition, the possible presence of intra-familial locus heterogeneity or compound heterozygous mutations might explain the phenotype of a small proportion of the families, and these would also be more amenable to whole exome sequencing than linkage or homozygosity mapping. In a recent study of Pakistani families with hearing impairment, intra-familial locus heterogeneity was detected in 15.3% of the families in their collection [14]. In addition, compound heterozygous mutations were identified in 2.7% of genetically resolved arRD families in a review of all published retinal degeneration cases in Pakistan [11]. Although homozygosity mapping has been proved effective, its main limitation is that familial locus heterogeneity or compound heterozygous mutations will be overlooked by this type of analysis.

In conclusion, as selection of both families and candidate loci was unbiased, this approach allows an accurate estimate of the contributions of various genes to arCC and arRD in the Pakistani population. The causative genes remain unidentified in about two thirds of arCC families and one third of arRD families in our study, making next-generation sequencing an ideal method for identification of new genes, especially since known candidate genes have largely been excluded in these families. Our results provide a key bridge between bench and bedside and should make genetic diagnose of arCC and arRD in patients more accessible and practical. This should greatly enhance the clinical genetic counseling, diagnosis, and early intervention of these disorders in the Pakistani population. These results also highlight the importance of analyzing the causative genes and their exons in different ethnic groups in a systematic and population-specific fashion.

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Genetics of Cataract in Asia: An Overview of Research in Congenital and Age-Related Cataract with Emphasis on Indian Populations

6

Chitra Kannabiran and Vanita Vanita

Abstract

Cataracts are a common cause of treatable visual impairment worldwide. They are classified based on the age at onset, which ranges from congenital (manifesting at birth or early childhood) to age-related (after the fourth decade of life). The etiology of each of these types of cataract differs with respect to the role of genetic and environmental factors in their development. Congenital hereditary cataracts are rare diseases that are single gene disorders which often run in families. Familial transmission is by autosomal dominant, recessive, and, less commonly, X-linked inheritance. Age-related cataracts are common causes of visual impairment in populations across the world and are triggered by various risk factors such as sunlight, smoke, diabetes, and age-related changes in the lens. In addition, genetic predisposition has been reported for age-related cataract, which has been associated with polymorphisms in various genes important for the lens.

Keywords

Eye lens • Age-related cataract • Congenital cataract • Crystallins • Gap junction channels • Mutations • Polymorphisms

6.1 Introduction

Cataract is an opacification of the eye lens and occurs due to genetic and/or environmental factors. Cataracts can arise at birth (congenital), early childhood (juvenile), before the age of 45 years (presenile), or afterward (senile or age-related cataracts). Among these different types of cataract, age-related cataract is a leading cause of blindness, especially with increase in

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the world's population along with an increase in longevity. Blindness due to cataract is estimated to affect over 20 million people worldwide [http://www.who.int/blindness/causes/priority/en/index1.html]. Though it is treatable by surgery, the rise in the number of cataracts throughout the world, coupled with barriers to accessing eye care services especially in developing countries, has placed a greatly increased demand on the requirement of cataract surgery in order to clear the burden of cataract-related blindness.

6.2 Epidemiology and Asian Perspective

Pediatric cataracts are documented to be responsible for more than 1 million childhood blindness in Asia [1]. In developing countries like India, 7.4–15.3% of childhood blindness is attributed to cataract [2]. Various population-based studies have shown that age-related cataract is also a common cause of visual impairment and blindness in adults worldwide. It constitutes about half of the burden of visual impairment among urban, rural, and migrant communities of Indians from different regions. In the state of Andhra Pradesh in South India, an epidemiological survey of the causes of blindness and visual impairment showed that age-related cataracts accounted for over 40% of this burden with a prevalence of 0.94% [3]. It is also a frequent cause of moderate visual impairment, defined as "visual acuity less than 6/18–6/60 or equivalent visual field loss in the better eye" [4]. In central India, cataract accounted for 75% of cases of visual impairment in adults [5]. A study on blindness in the rural population in South India found a similar prevalence of blindness due to age-related cataract of over 72% [6]. The relative contributions of genetic and environmental factors in the etiology of any disease can be assessed by looking at the frequency of the disease in an ethnic group that has migrated to a geographically distant location versus the same ethnic group in its country of origin. In this situation, the gene pool of the migrant population remains similar over generations to its native counterpart, assuming that no appreciable intercommunity marriage has occurred. A difference in the risk of any disease that occurs between two such populations is then attributable to the differences in their environments. One such study that examined cataract-related blindness in urban Indians living in Singapore found that it accounted for 60% or more of the total bilateral blindness in that region and prevalence of cataract was similar to that of the other Asians communities in Singapore but lower than the prevalence rates reported for Indians living in India. This suggests that environmental factors account for the difference in the frequency of cataract between the Indian communities in the two regions [7]. Another risk factor for cataract is severe dehydration, including that associated with one or more episodes of diarrhea. Case-control data on adults aged between 30–60 years in eastern and central India estimated that about 40% of blinding cataract may be attributable to dehydrational crises resulting from severe diarrheal disease [8]. In addition, cataract is associated with exposure to smoke and toxic chemicals [9].

6.3 Etiopathogenesis

The lens is made up of a very high concentration of proteins, which constitute about 30% of its entire mass. Proteins are present concentrations in the range of above 450 mg/ml within the lens. The bulk of the lens proteins consist of the crystallins, which are major structural proteins of the lens. The crystallins are soluble, cytoplasmic proteins, comprised of three major classes – the alpha-, beta-, and gamma-crystallins [10]. The α-crystallins make up 40% of human lens crystallin and consist of two related proteins, αA- and αB-crystallin. The αA-crystallin is essentially expressed in the lens, while αB-crystallin is fairly ubiquitous in its expression, being expressed in various tissues including the brain, muscle, lung, thymus, and kidney. Alpha-crystallin in the lens exists as a large complex, of about 800–1000 kDa, and is important in its function as a chaperone, maintaining the solubility of various lens proteins. It does this by binding to unfolded or denatured proteins and keeping them in solution, thus maintaining the

transparency of the lens. The beta- and gamma-crystallins are made up of a common structural unit, the Greek key motif. The Greek key motif consists of four-stranded antiparallel β-sheet structures. Two such motifs constitute a globular domain. The β-crystallins consist of two types based on overall charge. They are the acidic (βA1/A3, βA2, and βA4) and basic (βB1, βB2, and βB3) β-crystallins. The β-crystallins exist as homo- and heterodimers of basic and acidic subunits, organized into larger complexes of oligomeric proteins of 40–200 kDa. The gamma-crystallins are monomers of 20 kDa; the two globular domains of γ-crystallins are involved in intramolecular interactions, giving the domain a compact structure, and packing at very high densities in the lens. The globular domains of β-crystallins engage in intermolecular interactions between acidic and basic forms. Other proteins that are important for lens physiology are membrane proteins, gap junction proteins, and cytoskeletal proteins. Changes in any of the lens proteins can potentially lead to loss of ordered structure and the formation of lens opacities. Pathogenic mutations in various genes encoding the aforementioned proteins are associated with congenital hereditary cataracts in families of Indian origin.

Age is a universal risk factor for cataract. The lens undergoes continuous changes in its biochemical and biophysical properties throughout life, and these lead to light scattering, coloration, and stiffness of the lens with age. Increase in light scattering steeply increases after the fourth decade with a greater degree of scattering in the deep cortex as compared with the superficial cortex and nucleus [11]. Another age-related change in the lens is increasing stiffness starting from birth onward, affecting the lens nucleus more than the cortex. This decreases its deformability and accommodative power and leads to presbyopia. Changes in the properties of the lens as a function of age are accompanied by various alterations in lens proteins such as oxidation, cross-linking, proteolysis, and denaturation. Posttranslational modifications that take place in lens proteins as a function of age are thiolation, deamidation, glycation, carbamylation, *cis*-methylation, phosphorylation, and acetylation, primarily involving the crystallins [12]. Photochemical studies on lens crystallins showed that beta- and gamma-crystallins, particularly, undergo changes in conformation and structure, resulting in colorization and insolubility of these proteins. Modifications such as cross-linking of proteins occur due to reactive oxygen species-induced oxidation of tryptophan or tyrosine residues. Most light below 295 nm is absorbed by the cornea. The molecules that absorb light above this wavelength are tyrosine and tryptophan (at 275 nm and 290 nm, respectively). Photolysis of tryptophan generates N-formyl kynurenine, which is further converted to kynurenine and other products. Metabolites of tryptophan are capable of generating singlet oxygen and free radicals. The free radicals are highly reactive and capable of covalently modifying proteins. Oxidative attacks on unfolded or otherwise modified crystallins result in cross-linking, insolubility, and the formation of high-molecular-weight aggregates. Sugar-related cataracts arise from osmotic imbalances within the lens that are caused by excessive accumulation of sugars, such as glucose, galactose, and pentoses. The presence of sugars leads to nonenzymatic glycation of lens proteins [13]. As compared to congenital cataract, age-related cataract is multifactorial in its etiology, and its formation and progression rely on the presence of one or more of several risk factors. Smoke is another established etiologic factor for age-related cataract, and numerous studies have brought out the increase in risk of cataract among smokers. A population-based cross-sectional epidemiologic study was carried out in rural and urban men and women smokers of beedis, cigars, and cigarettes. A significantly higher prevalence of nuclear and cortical cataracts was found among smokers. Compared with never-smokers, "heavy" cigarette smokers had a higher prevalence of nuclear cataract, followed by cortical cataract [9].

6.4 Clinical Features

Cataracts are characterized based on the location of the opacity and whether total or partial. The

major types of age-related cataract are cortical, nuclear, and posterior subcapsular. Various systems of classification and grading of cataracts have been developed on the basis of the type and degree of opacification involved. These systems rely on the use of slit lamp evaluation of cataracts or by assessment of slit lamp photographs taken on retro-illumination. Different systems of grading cataracts include the Lens Opacities Classification System (LOCS), Wisconsin Cataract Grading System, and others [14]. The LOCS system consists of LOCS I, LOCS II, and LOCS III, which have evolved successively to incorporate modifications in the grading and description of lens opacities. Under the LOCS II system, the categories of cataract are nuclear opalescence (NO), nuclear color (CO), cortical cataract (C), and posterior subcapsular cataract (P). The LOCS III system [15] is a more recent version after LOCS II in grading cataracts and is designed to have better reproducibility. LOCS classification systems are reported to have clinical utility in optimizing the surgical parameters of surgery such as time and power of phacoemulsification, which may be correlated to the cataract grade, especially for nuclear cataracts.

On the basis of morphology, congenital cataract has been broadly classified as polar cataract involving either the anterior or posterior or both the poles of the lens, anterior or posterior subcapsular cataract, zonular cataract including nuclear cataract, sutural cataract, cerulean (blue dot) cataract, and membranous or capsular cataracts [16], although various types and subtypes and many unique forms have also been documented.

6.5 Genetic Aspects

6.5.1 Age-Related Cataracts

It is well recognized that genetics plays a role in the etiology of ARCs as well, in addition to other external agents [10]. Analysis of ARC for associated genetic factors generally involves case–control studies of associations with polymorphisms or SNPs at selected genomic loci or the screening of candidate genes for sequence changes or mutations present in affected cases but absent in normal controls. Genes that are evaluated for their association to ARC are chosen as candidates because they are recognized as important for the normal structure and transparency of the lens or because they are already associated with cataract (both congenital and age-related) or associated with other age-related eye diseases. Among these, genes functioning in DNA repair and oxidative stress pathways have been commonly studied for variations that are associated to age-related cataracts. This is because the antioxidant capacity of the lens declines with age. The redox capacity of the lens is maintained by various molecules such as ascorbic acid, as well as thiols, among which the level of glutathione (GSH) is critical. The level of reduced GSH decreases almost linearly with age, along with an increase in the oxidized form (GSSG) [11]. The generation of GSH in turn requires enzymes such as glutathione S-transferases (GST), glutathione reductase (GR), and glutathione peroxidase, which are also reported to decline in activity in aging lenses. With increasing age of the lens, its redox capacity is thus compromised, making it susceptible to oxidative stress. Reactive oxygen species (ROS) are generated in the lens epithelium and the superficial fiber cells, and ROS production in the lens may exceed the capacity to neutralize them. ROS may cause oxidative damage to mitochondrial DNA and nuclear DNA. In normal physiologic conditions, most oxidative DNA lesions are rapidly repaired. Cumulative DNA damage due to oxidative stress, coupled with an age-related decline in activity of DNA repair enzymes, leads to damage of cell membranes and cataractous changes in the lens. Genes investigated in case–control associations on Indians with ARC are the DNA repair genes particularly the xeroderma pigmentosum complementation group (XPD) and X-ray complementing group1 (XRCC1) loci, GST including the *mu* 1 (*GSTM1*) and the *theta* 1 (*GSTT1*) genes, the receptor tyrosine kinase gene ephrin A2, lens structural protein genes (crystallins such as *CRYAA*), and a few others [17–19]. A larger population-based study

that involved patients from two locations in North and South India evaluated SNPs in the ephrin A2 gene and found association of two SNPs with cortical cataract and posterior subcapsular cataract in this population [20]. Ephrin A2 was considered as a candidate since it was linked with cortical cataracts in an earlier genome-wide study on participants from the Beaver Dam Eye Study (BDES) that recruited people from the state of Wisconsin in the USA.

Likewise, there have been several studies on the genetics of age-related cataract in various Asian populations, particularly the Han Chinese, focused on the role of DNA repair genes [21], glutathione peroxidase (*GPX*), lens membrane proteins (*MIP*, *GJA8*) [22], and oxidative stress response genes required for neutralizing oxygen-free radicals (such as superoxide dismutase, catalase). Among these, one study included participants that were drawn from the Jiangsu Eye Study, a population-based epidemiologic study in China [21]. Sample sizes across various association studies have generally ranged from 100 to 400 cases and an equal number of controls. Associations of SNPs at various loci are reported with the caveat that sample sizes generally employed may not be adequate for the detection of small to moderate genetic effects. The robustness of these associations needs to be tested in larger populations. Thus, there are at times conflicting data on the associations of specific genes with ARC for a particular population. Apart from sample sizes, inconsistencies could arise due to stratification of the population of cases or controls and in situations in which controls are not ethnically matched to cases. Meta-analysis of data can be employed to increase the power to detect association by combined analysis of data from different studies that have used comparable methods. Such meta-analyses of genetic association studies on ARC have pointed to population-specific associations of markers with the disease (see, e.g., [23]). A meta-analysis of various Asian studies (including those from India, China, and Turkey) on the role of DNA repair genes in ARC showed the genetic polymorphisms of *XRCC1* (Arg399Gln) and *XPD* (Lys751Gln) to be significantly associated with increased susceptibility to age-related cataracts [24].

6.5.2 Congenital Cataract

Congenital cataracts are genetically heterogeneous and have autosomal dominant, recessive, and X-linked modes of inheritance. Various genes for congenital cataracts have been identified in Indian families having autosomal dominant or autosomal recessive cataracts. The various genes known till date, mutations, associated phenotypes, and other details are shown in Table 6.1. Padma et al. [28] analyzed a South Indian autosomal dominant congenital cataract (ADCC) family with ten members in three generations affected by Y-sutural cataract. After excluding the previously known candidate loci, i.e., Duffy blood group locus, the y-crystallin gene cluster, haptoglobin locus, glutathione reductase, αA-crystallin, and βB2- and βB3-crystallin genes, authors reported linkage with microsatellite markers at chromosome 17 with a maximum LOD score of 3.9 at $\theta = 0$ at D17S805. By multipoint and haplotype analysis, a novel cataract locus was confined to an interval of 17 cM between markers D17S799 and D17S798 at 17q11.1–12 in proximity to a candidate gene crystallin βA1/A3. Kannabiran et al. [29] sequenced βA1/A3 crystallin gene and identified a novel G to A transition in the 5′ (donor) splice site of exon 3 resulting in splice site mutation in all the affected members of this Y-sutural cataract family.

In a seven-generation family of North Indian origin with 74 individuals in seven generations affected by ADCC with a unique cataract phenotype, i.e., "central pouch-like" cataract with sutural opacities, Vanita et al. [54] excluded previously known cataract loci at chromosomes 1, 2, 3, 10, 12, 13, 14, 16, 17, 19, 21, and 22. In a genome-wide search using 360 markers, linkage was identified to microsatellite markers on chromosome 15 (maximum LOD score of 5.98 at $\theta = 0$ with marker D15S117). By multipoint and haplotype analyses, the cataract locus in this ADCC family has been placed in a 10-cM

Table 6.1 Loci/genes reported with different phenotypes of congenital cataract in analyzed Indian families

Locus	Gene family/ gene and OMIM no.	Exon/ intron	Nucleotide change	Amino acid change	Phenotype	Associated eye/other anomaly	Inheritance pattern/no. of affected members	Origin of the family	References
Crystallins									
	Alpha-crystallins								
21q22.3	**CRYAA [123580]**	Exon 1	c.34C>T	p.R12C	Variable, lamellar, nuclear, dot-like	Microcornea	AD	South India	[25]
		Exon 1	c.61C>T	p.R21W	Anterior polar, nuclear, lamellar	Microcornea	AD	South India	[25]
		Exon 1	c.160C>T	p.R54C	Y-sutural	Microcornea	AD	South India	[25]
		Exon 2	c.292G>A	p.G98R	Progressive, ringlike		AD	South India	[26]
		Exon 2	c.346C>T	p.R116C	Fan-shaped nuclear	Microcornea	AD	North India	[27]
11q22.1-q23.2	**CRYAB [123590]**	Exon 3	c.511G>A	p.A171T	Lamellar		AR	South India	[25]
	Beta-crystallins								
17q11.2-q12	**CRYBA1/ A3 [123610]**	IVS3	c.215+1G>A		Zonular sutural		AD	South India	[28, 29]
		IVS3	c.215+1G>A		Lamellar, floriform		AD	South India	[25]
		IVS3	c.215+1G>A		Lamellar		AD	South India	[25]
22q12.1	**CRYBA4 [123631]**	Exon 4	c.206 T>C	p.L69P	Cataract	Microphthalmia	AD	South India	[30]
		Exon 4	c.281 T>C	p.F94S	Lamellar		AD	South India	[30]
		Exon 4	TAC>AAC	p.Y67N	Nuclear cataract		Sporadic	North India	[31]
22q12.1	**CRYBB1 [600929]**	Exon 3	GAC>AAC and GAA>AAA (both in the same patient)	p.D85N and p. E75K	Nuclear cataract		Sporadic	North India	[31]
		Exon 6	GAA>AAA	p.E155K	NS		Sporadic	North India	[31]
22q11.23	**CRYBB2 [123620]**	Exon 4	c.177G>C	p.W59C	Total		AD	South India	[32]
		Exon 2	c. G54A		Zonular cataract		AD;IP	South India	[32]
		Exon 6	c.452G>C	p.W151C	Central nuclear		AD	South India	[33]
		Exon 6	c.463C>T and c.471C>T	p.Q155X	Sutural cerulean		AD	North India	[34]
		Exon 6	c.463C>T	p.Q155X	Cortical, pulverulent		AD	India	[25]
	Gamma-crystallins								
2q33-q35	**CRYGB [123670]**								

2q33-q35	**CRYGC [123680]**	Exon 2	c.143G>A	p.R48H	Nuclear pulverulent		AD	North India	[35]
		Exon 3	c.502C>T	p.R168W	Lamellar		AD	South India	[36]
		Exon 3	c.502C>T	p.R168W	Lamellar		AD	India	[25]
2q33-q35	**CRYGD [123690]**	Exon 2	c.70C>A	p.P23T	Lamellar		AD	South India	[36]
		Exon 2	c.70C>A	p.P24T	Aculeiform		AD	North India	[37]
		Exon 2	c.229C>A	p.R77S	Juvenile, anterior polar coronary		AD	South India	[38]
		Exon 2	c.418C>T	p.R140X	Nuclear		AD	India	[25]
		Exon 2	c.470G>A	p.W156X	Central nuclear		AD	South India	[36]
3q25-qter	**CRYGS [123730]**	Exon 2	c.116C>G	p.S39C	Sutural, lamellar, progressive		AD	South India	[25]
		Exon 2	c.124G>A	p.V42M	Opalescent, central nuclear		AD	North India	[39]
Connexins (gap junction channels)									
1q21.1	**GJA8 [600897]**	Exon 2	c.131T>A	p.V44E	Total	Microcornea, mild myopia	AD	South India	[40]
		Exon 2	c.134G>C	p.W45S	Jellyfish-like	Microcornea	AD; five affected in three generations	North India	[41]
		Exon 2	c.235G>C	p.V79L	Full moon with Y-sutural opacities		AD	North India	[42]
		Exon 2	c.262C>A	p.P88Q	Balloon-like, sutural		AD	India	[43]
		Exon 2	c.649G>A	p.V196M	congenital		AR	India	[44]
		Exon 2	c.593G>A	p.R198Q	Posterior subcapsular	Microcornea, mild myopia	AD	South India	[40]
		Exon 2	c.658C>T	p.P199S	Congenital		AD	India	[44]
		Exon 2	c.608insA	p.T203NfsX47	Total	Nystagmus	AR	South India	[45]
		Exon 2	c.84G>A	p.V28V branch point			AD	India	[44]
		Exon 2	c.842T>C	p.L281C	Lamellar/zonular	Nystagmus	AD	North India	[35]
13q11-q12	**GJA3 [121015]**	Exon 1	ATG>GTG	p.M1V	Anterior polar cataract		Sporadic	North India	[31]
		Exon 2	c.56C>T	p.T19M	Posterior polar		AD	South India	[32]
		Exon 2	c.82G>A	p.V28M	Total, anterior capsular, cortical		AD	South India	[46]
		Exon 2	c.98G>T	p.R33L	Granular embryonal		AD	North India	[47]
		Exon 2	c.226C>G	p.R76G	Total		AD	South India	[46]

(continued)

Table 6.1 (continued)

Locus	Gene family/ gene and OMIM no.	Exon/ intron	Nucleotide change	Amino acid change	Phenotype	Associated eye/other anomaly	Inheritance pattern/no. of affected members	Origin of the family	References
		Exon 2	c.260C>T	p.T87M	"Pearl boxlike"		AD	North India	[48]
		Exon 2	c.589C>T	p.P197S	Lamellar		AD	India	[44]
Membrane proteins									
12q13	**MIP [154050]**	Exon 2	c.494G>A	p.G165D	Lamellar		AD	South India	[31]
19q13.4	**LIM2 [154045]**	Exon 4/5	c.462G>A	p.G154E	Congenital	Nystagmus, amblyopia	AR	India	[49]
Beaded filament proteins									
20p11.23-p12.1	**BFSP1 [603307]**	Exon 6	c.736-957del	p.T246del74fsX6	Cortical progressive, juvenile onset		AR	India	[50]
Transcription factors									
16q22-q23	**MAF [177075]**	Exon 1	c.890A>G	p.K297R	Cerulean	Microcornea	AD	North India	[51]
Others									
	FTL [134790]	Exon 1 (IRE)	c.-168G>A		Y-sutural	Hyperferritinemia	AD	India	[52]
Xp22.13	**NHS [300457]**	Exon 1	c.115C>T	p.Q39X	Cortical cuneiform, sutural, corolliform, blue dot, posterior subcapsular	Microcornea	XL	South India	[53]
15q21-22	**CTRCT25 605728**				Central "pouch-like" cataract with sutural opacities			North India	[54]

Notes: *AD* autosomal dominant, *AR* autosomal recessive, *XL* X-linked, *IP* incomplete penetrance, *NS* not specified

region, between D15S209 and D15S1036 and closely linked to D15S117, corresponding to the q21-22 region of chromosome 15. By mutation analysis, candidate genes *FBN-1*, *FGF-7*, and *RORA* localized at the mapped interval at 15q21-22 have been excluded.

In an ADCC family with 33 members in five generations affected by a unique form of sutural cataract with punctate and cerulean opacities, Vanita et al. [34], in a candidate gene approach after excluding previously known cataract loci at chromosomes 1, 2, 12, 13, 14, 16, 17, and 19, detected linkage with marker D22S315, with an LOD score of $Z_{max} = +8.5$ at $\theta_{max} = 0.05$ at 22q11.2 that harbors cluster of β-crystallin genes, *CRYBA4*, *CRYBB1*, *CRYBB2*, and *CRYBB3*, and a pseudogene *CRYBP1*. Mutation screening in *CRYBB2* (highly expressed in the lens cells) indicated a previously reported c.475C>T; p.Q155X mutation (linked with cerulean cataract and Coppock-like (central zonular pulverulent) cataract in two ADCC families of English and Swiss ancestry) and a previously unreported additional substitution, i.e., c.483C>T. Both these changes completely segregated in all the affected members of this Indian family. The alignment of the *CRYBB1*, *CRYBB2*, *CRYBB3*, and *CRYBP1* sequences showed the alterations p.Q155X and c.483C>T in the *CRYBB2* mutant to be identical to the "normal" sequence in the *CRYBP1* pseudogene that are localized just 228 kb apart at 22q11.2 and mutant allele carried >9 bp to <104 bp of pseudogene-like sequence. Thus, the most likely explanation that we gave is gene conversion between *CRYBB2* exon 6 and its homologous *CRYBP1* sequence led to the pseudogene-like alteration in the *CRYBB2* gene that also modified the cataract phenotype in our analyzed Indian family.

Santhiya et al. [36] performed DNA sequence analysis in four candidate genes CRYGA-D in seven South Indian families with nonsyndromic ADCC and identified three novel mutations, i.e., p.P23T in CRYGD, and p.R168W in CRYGC for lamellar cataract in one family each and p. W156X in CRYGD in third family with central nuclear cataract. In nine congenital cataract families from South Indian, Santhiya et al. [33] undertook mutation screening by DNA sequence analysis in candidate genes, *CRYGA-D*, *CRYBB2*, and *GJA8*. Authors reported a novel mutation, i.e., p.W151C in exon 6 of *CRYBB2* in one of the analyzed four-generation ADCC family having nine members affected by central nuclear cataract. Talla et al. [55] analyzed the expressed wild-type and mutant R168W CRYGC protein and predicted mutant CRYGC to be similar to the wild-type molecule in conformation and structural stability, however, different in its ability to aggregate and light scattering.

Devi et al. [46] by PCR-SSCP analysis tested 60 probands from South India (belonging to 32 ADCC families, 18 ARCC families, and 10 with uncertain inheritance pattern) with nonsyndromic congenital cataract for mutations in the *GJA3*, followed by sequence analysis of samples which indicated an electrophoretic shift. In one ADCC family with three affected members by total cataract, authors reported a novel p.R76G mutation and in another family with seven affected members in four generations by total cataract with delayed onset a novel p. V28M substitution that also segregated in two unaffected members and was absent in 400 tested controls.

A four-generation South Indian family, with eight affected males who inherited X-linked developmental lens opacity and microcornea and some members with mild to moderate non-ocular clinical features suggestive of Nance–Horan syndrome (NHS), was analyzed by Ramprasad et al. [53]. Using microsatellite markers at X-chromosome (an interval of 10 cM), by multipoint analysis authors documented the peak LOD of 4.46 at *DSX987* in proximity to gene for the *NHS*. By mutation screening 115C>T, substitution in exon 1 resulting in conversion of glutamine to stop codon (p.Q39X) has been revealed in all the affected males and carrier females of this family and in none of the unaffected family members or tested control subjects.

In an ADCC family from North India, in three generations with 12 affected members with cerulean cataract and six affected members having

microcornea, by linkage analysis using >100 microsatellite markers for known cataract loci, linkage was detected at chromosome 16 - (two-point LOD score of 3.9 at $\theta = 0.0$ with D16s3049). Multipoint and haplotype analysis placed the cataract locus to a 15.3-cM region between markers D16S518 and D16S511 at 16q23.1. Sequencing of the candidate gene MAF, at the linked region, revealed a novel heterozygous missense mutation, i.e., c.890A>G; p.K297R, in the basic region (BR) of the DNA-binding domain that co-segregated completely with disease and in none of the unaffected family members or in 106 unrelated control subjects [51].

The effect of three different mutations reported in the BR of the DNA-binding domain of MAF was tested by heterologous expression, p.R288P, associated with pulverulent cataract in an English family; K297R, with cerulean cataract in our analyzed Indian family; and R299S, for the most severe phenotype of congenital cataract and microcornea syndrome detected in another three-generation English family. Taking *CRYAA*, *CRYBA4*, *CRYBA1*, and *CRYGA* as c-Maf target genes (with conserved half-site MAF-responsive elements) in their promoter/UTR regions, and four MAF expression constructs one with the wild-type MAF and with three mutants, i.e., R288P, K297R, and R299S, we performed luciferase reporter assays. The mutation R288P significantly reduced the expression of the CRYGA and CRYBA1 constructs but had no significant effect on the other two constructs. K297R did not lead to a significant reduction in expression of any of the four constructs, although with a reduced expression especially for the CRYGA construct. R299S was associated with the most severe overall effect on the transactivation of the four crystallin expression constructs; we further suggested that differential effects of mutations on the transactivation potential of c-Maf may be a molecular correlate of the striking genotype–phenotype correlations seen in cataract forms caused by mutations in the *MAF* [56].

In a South Indian family, with four members in three generations having nonsyndromic presenile cataract of progressive type, Santhiya et al. [26], by mutation screening in three candidate genes, i.e., *CRYGC*, *CRYGD*, and *CRYAA,* identified, i.e., c.292G>A; p.G98R change in exon 2 of CRYAA. Singh et al. [57] further reported that the mixed oligomers of wild-type and G98R αA-crystallin showed properties dominated by those of the mutant protein in structural aspects, oligomeric size, urea-induced unfolding, and, more importantly, the chaperone activity.

By genome-wide linkage analysis using >400 markers in combination with multipoint LOD score and haplotype analysis in 27 members of a North Indian family, with 13 members in four generations affected with Y-sutural congenital cataract, we reported a maximum two-point LOD score of 6.37 at θ=0.00 with marker D19S879 [52]. Multipoint and haplotype analysis placed the cataract locus to a 5.0 cM interval between marker D19S902 and D19S867, in proximity to the gene for L-ferritin light chain (*FTL*) on chromosome 19q13.3. Hematological tests performed in two affected individuals showed very high levels of serum ferritin without iron overload leading to the diagnosis of hyperferritinemia–cataract syndrome. Mutation screening in *FTL* localized at the mapped interval indicated a G>A substitution at position 32 (c.-168G>A) in a highly conserved three-nucleotide motif that forms a loop structure in the iron-responsive element (IRE) in the 5′-untranslated region that co-segregated completely with the disease phenotype.

In another ADCC family of North Indian origin having 15 members in three generations, affected with bilateral cataract that gave the appearance of "full moon" with Y-sutural opacities, the cataract locus was mapped in proximity to the *GJA8* gene at 1q21. Mutation screening in *GJA8* identified a novel transversion, i.e., c.235G>C, resulting in p.V79L. change [42].

In a South Indian family with >20 members in four generations affected by congenital lamellar cataract, Billingsley et al. [30] in a genome-wide linkage analysis identified a maximum LOD score of 3.20 ($\theta = 0.001$) with marker D22S1167 in proximity to the β-crystallin gene cluster on chromosome 22. By direct sequencing

analysis of the coding sequence of the four β-crystallin gene cluster (*CRYBB2, CRYBB3, CRYBB1,* and *CRYBA4*), authors identified a novel heterozygous c.317T>C transition (p. F94S) in *CRYBA4* exon 4.

In a North Indian family, ten members in four generations were affected with a unique form of fan-shaped cataract–microcornea syndrome. In a genome-wide screening by two-point linkage analysis using >400 microsatellite markers in combination with multipoint LOD score and haplotype analysis, the cataract–microcornea locus was mapped to a 23.5-cM region on chromosome 21q22.3 [27]. Bidirectional sequencing of the candidate gene *CRYAA* at the mapped interval revealed a heterozygous C>T transition at codon 116 (CGC-TGC), resulting in the substitution of the highly conserved arginine by cysteine (p. R116C) that segregated completely with the disease in this family.

Devi and Vijayalakshmi [40] by PCR-SSCP and sequence analysis tested 60 probands with congenital or developmental cataract from South India for mutations in the *GJA8* gene. Authors identified a novel missense mutation, i.e., p. V44E, in one family having two members affected with total congenital cataract, microcornea, and variable myopia. In another family with three affected members having bilateral developmental cataract, microcornea, and mild myopia, another novel missense mutation, i.e., p.R198Q, was detected.

Ponnam et al. [45] analyzed a South Indian ARCC family with eight candidate genes by PCR-SSCP and sequence analysis and reported a frameshift mutation (c.670insA; p. Thr203AsnfsX47) of GJA8 and demonstrated for the first time the involvement of connexin 50 in recessive cataract.

In a North Indian ADCC family with 18 members in four generations affected with embryonal cataract, Guleria et al. [47], in a genome-wide scan using the GeneChip Human Mapping 10K Array, mapped the disease locus at chromosome 13q11 in the vicinity to gene for GJA3. By sequence analysis, a novel mutation, i.e., p.R33L, was identified that segregated completely with the disease phenotype. By mutation screening in four ADCC and three ARCC families in GJA3 gene, Guleria et al. [48] reported a novel mutation, i.e., p.T87M, in an ADCC family, with a unique "pearl boxlike" cataract.

In a genome-wide scan in a consanguineous family of Indian origin with autosomal recessive developmental cataract, Ramachandran et al. [50] performed two-point linkage analysis with 382 microsatellite markers and detected linkage to markers on chromosome 20q, between D20S852 and D20S912, with a maximum LOD score of 5.4 with D20S860. By mutation screening in the candidate gene, i.e., BFSP1, at the mapped interval, authors reported a 3343-bp deletion including exon 6 (c.736-1384_c.957-66 del) predicted to result in a shift of the open reading frame. This is the first report of a mutation in the *BFSP1* gene associated with human inherited cataracts.

In 60 South Indian families, Devi et al. [25] performed mutation screening in ten crystallin genes, i.e., *CRYAA, CRYAB, CRYBA1, CRYBA4, CRYBB1, CRYBB2, CRYBB3, CRYGC, CRYGD*, and *CRYGS* by PCR-SSCP and sequencing. Authors reported causative mutations in ten of the analyzed families including six missense mutations (p.R12C (for lamellar, nuclear), p. R21W (for anterior polar, nuclear, lamellar phenotype), p.R54C (with Y-sutural cataract) in CRYAA, p.A171T (a novel mutation with lamellar cataract) in CRYAB, p.R168W (for lamellar cataract) in CRYGC, p.S39C (a novel mutation with sutural cataract) in CRYGS), two nonsense mutations (Q155X (for cortical, pulverulent) in CRYBB2, p.R140X (a novel mutation with nuclear cataract) in CRYGD), and one splice mutation, seen in two families (IVS3+1G>A (with lamellar cataract) in the CRYBA1 gene).

Vanita et al. [41] in an ADCC family of North Indian origin having five members in three generations affected by unique jellyfish-like cataract in association with microcornea performed mutation screening in the candidate genes, *CRYAA*, *CRYBB1*, *MAF*, *GJA3*, and *GJA8*. A novel heterozygous mutation, i.e., c.134G>C; p.W45S, was observed that segregated completely with the disease phenotype.

Ponnam et al. [49] analyzed 40 congenital cataract families with ten candidate genes (six crystallin genes, two connexin genes, *LIM2*, and *HSF4*) by PCR-SSCP and sequence analysis and reported a missense mutation (p.G154E) in LIM2 gene linked with ARCC in a family with total cataract.

In another ADCC family having three members in two generations affected by "balloon-like" cataract with prominent Y-sutural opacities, we performed mutation screening in nine candidate genes, *CRYAA*, *CRYBA1*, *CRYBB2*, *CRYGA*, *CRYGB*, *CRYGC*, *CRYGD*, *GJA3*, *and GJA8*, by sequence analysis. A heterozygous c.262C>A change in the GJA8 gene localized at 1q21 that resulted in the replacement of a highly conserved proline by glutamine (p.P88Q) was observed in all the affected family members [43].

In an ADCC family from North India having seven affected members in three generations by opalescent-type cataract, we performed linkage analysis for the already known candidate gene loci in combination with mutation screening by bidirectional sequencing. Sequencing the candidate gene, crystallin gamma S (CRYGS), at chromosome 3q26.3-qter showed a novel heterozygous c.176G>A change that resulted in the replacement of a structurally highly conserved valine by methionine at codon 42 (p.V42M) [39]. Vendra et al. [58] cloned and expressed the mutant and wild-type gamma S cDNA to analyze the secondary and tertiary structures of the expressed proteins. Authors reported that the replacement of valine at position 42 by the longer and bulkier methionine disturbs the compact β-sheet core packing topology in the N-terminal domain of the molecule, exposes nonpolar residues thereby increasing the surface hydrophobicity, and weakens the stability of the protein, thus promoting self-aggregation leading to light scattering particles.

Vendra and Balasubramanian [59] analyzed the structural and aggregation behavior of the human γD-crystallin mutant E107A, associated with congenital nuclear cataract in two siblings [60], and reported that mutant γD-crystallin protein did not affect its conformation and stability but resulted in significant reduction in solubility and generation of light scattering aggregate particles in vitro and in situ when introduced into HeLa cell lines.

Santhiya et al. [32] screened five ADCC families for possible mutations with candidate genes, *CRYAA*, *CRYBB2*, *CRYGC*, *CRYGD*, *GJA3*, *GJA8*, *PITX3*, and *HSF4*. Authors identified three novel mutations in three families, p.W59C in *CRYBB2* for total cataract, p. T19M in *GJA3* with posterior polar cataract, and the third one with reduced penetrance in *CRYBB2* (c.G54A) in a proband with zonular cataract.

Roshan et al. [38] screened 48 members from 17 families and 148 sporadic cases of childhood cataract with candidate genes, *CRYAA*, *CRYBB2*, *CRYGA*, *CRYGB*, *CRYGC*, *CRYGD*, *GJA3*, *GJA8*, and *PAX6*, by PCR-SSCP and DNA sequence analysis. Authors reported a novel substitution (p.R77S) in *CRYGD* in an autosomal dominant family having three members affected with juvenile anterior polar coronary cataract.

Thirty congenital cataract cases below 3 years of age from North India were tested for mutation in candidate genes *CRYAB*, *CRYGC*, *CRYGD*, and *GJA8* [35]. Authors documented a total of six variations, two novel (CRYGC; p.R48H (for autosomal dominant nuclear pulverulent cataract) and GJA8; p.L281C with autosomal dominant lamellar/zonular cataract) and four previously reported (CRYAB; rs11603779T>G (with progressive lamellar cataract), GJA8; p.L268L (for anterior polar cataract), CRYGD; p.R95R (for lamellar cataract), and c. T564C (for nuclear cataract)). Both the novel changes, in CRYGC and GJA8, were found in 16.6% of their analyzed patients, and already reported nucleotide alterations (CRYGD; p.R95R and c.T564C) were found in 90% of their analyzed patients.

Vanita and Singh [37] in two ADCC families, with, respectively, twenty (by bilateral aculeiform type cataract) and four affected members (with bilateral granular nuclear cataract), undertook mutation screening in 23 candidate genes including crystallins, gap junctional channels, connexins, beaded filament chain proteins, *MIP*, *LIM2*, and *MAF* and in *TMEM114*, *CHMP4B*, and *EPHA2* by sequence analysis. p.Pro23Thr in

CRYGD was observed for aculeiform type cataract, thus expanding the genetic heterogeneity for this phenotype.

In an attempt to identify underlying genetic defect in three families from South India with inherited cataract of maternal origin, Roshan et al. [61] identified 72 mitochondrial DNA (mtDNA) variations in these three tested families. Four variations were reported to be specific for cataract patients of the first family, six for the second family, and one for the third family.

Ponnam et al. [44] screened 40 Indian families with ADCC and ARCC in ten candidate genes by PCR-SSCP and sequence analysis. Authors reported four novel sequence changes that co-segregated with the disease phenotype. These changes included a homozygous missense change, c.649G>A (p.V196M) in *GJA8* in a family with ARCC; two heterozygous missense changes, c.658C>T (p.P199S) in *GJA8* and c.589C>T (p.P197S) in *GJA3* in two families with ADCC; and a silent change, c.84G>A (p. V28V), predicted to result in the creation of a new potential branch point in *GJA8* in one family with an ADCC. Authors further predicted that that connexin gene (*GJA8* and *GJA3*) mutations occur in approximately 10% (4/40 families) of families with congenital hereditary cataracts in a population from South India.

In 100 sporadic congenital cataract cases from North India, Kumar et al. [31] tested candidate genes, *CRYAA*, *CRYAB*, *CRYGs*, *CRYBA1*, *CRYBA4*, *CRYBB1*, *CRYBB2*, *CRYBB3*, *BFSP1*, *GJA3*, *GJA8*, and HSF4, by sequence analysis. Authors identified 18 nucleotide variations, 14 in the crystallin genes, one in *GJA3*, and three in *BFSP1* of which five variations (*CRYBA4*, p. Y67N; *CRYBB1*, p.D85N; *CRYBB1*, p.E75K; *CRYBB1*, p.E155K; and *GJA3*, p.M1V) were predicted to be pathogenic.

In an ADCC family from South India with four affected members in three generations with congenital lamellar cataract, Senthil Kumar et al. [31], by candidate gene screening, reported a novel mutation (c.494G>A; p.G165D) in the *MIP* that segregates with a congenital lamellar cataract in a South Indian family. By functional assay, authors predicted improper trafficking of AQP0-G165D and loss of water channel function.

6.6 Molecular Biology

Molecular studies on cataract have addressed primarily the analysis of gene expression in lens epithelia from cataractous lenses as compared with normal control lenses. The lens epithelium is responsible for maintaining the homeostasis of the lens and carries out the bulk of the transcription in the lens. Changes in gene expression in response to aging or environmental insults would therefore be reflected in the lens epithelium. The genes that are altered in age-related cataractous lenses include important lens proteins such as alpha-crystallin, DNA repair proteins, members of the glutathione pathway, and those involved in oxidative stress responses. Some of these loci are subject to epigenetic regulation involving methylation of CpG islands, which is detectable with bisulfate-specific PCR and sequencing; hypermethylation of a given set of genes is associated with a corresponding decrease in gene expression in lenses with age-related cataract [22, 62, 63].

6.7 Summary

While congenital hereditary cataracts are rare and manifest in early childhood or adolescence, age-related cataract is a major cause of visual impairment in the older population worldwide. Specific alterations in various genes important for normal structure and function of the lens are involved in the pathogenesis of congenital hereditary cataracts. Genes that are associated to various forms of congenital cataracts include those encoding lens structural proteins, gap junction proteins, membrane proteins, and transcription factors. Exposures to environmental risk factors including sunlight, ultraviolet radiation, and smoke are associated with age-related cataract, in addition to the age-related processes in the lens. These latter processes involve posttranslational

changes in lens proteins, denaturation and fragmentation of proteins in response to oxidative stress, cross-linking and formation of protein aggregates, and decrease in lens elasticity. Genetic risk factors that are implicated by association studies include genes involved in DNA repair processes, oxidative stress, and those encoding lens structural proteins. The role of these pathways in cataract is also supported by studies that have demonstrated changes in expression profiles of these genes in cataractous lenses. Although current evidence suggests the contribution of various genes for DNA repair, redox pathways, and lens structural proteins to the etiology of age-related cataracts, larger studies on the genomics of cataract for each population as well as further analysis of these loci for identification of causative variants are required to arrive at a better understanding of the molecular etiology of age-related cataract.

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Genetic Analysis of Families with Retinal Dystrophies

7

Qingjiong Zhang

Abstract

Hereditary retinal dystrophies are the common and severe forms of genetic eye diseases. They are clinically and genetically highly heterogeneous. Since the identification of the RB1 gene and the visual pigment genes in 1986, a number of molecular genetic study on these diseases lead to the identification of over two hundred genes responsible for such diseases when mutated in the last three decades. Understanding the mutation spectrum and frequency across these genes as well as determining the pathologic role of a mutation shall be a prerequisite in clinical gene diagnosis and potential gene-based intervention. In this chapter, we would like to describe our preliminary analysis of the mutation spectrum and frequency of all these genes in several cohorts of patients with retinitis pigmentosa, Leber congenital amaurosis, or cone or cone-rod dystrophy from a single institute. The importance of family analysis has been emphasized in the identification of the molecular defects for these diseases. Hopefully this chapter will bring you some uncommon information that might be useful for those physician scientists who are ready to conduct routine clinical gene diagnosis and gene-based intervention from the point of family analysis.

Keywords

Hereditary retinal dystrophies • Retinitis pigmentosa • Leber congenital amaurosis • Cone-rod dystrophy • Molecular genetics • Gene • Mutation spectrum and frequency • Phenotype • Family analysis • Exome sequencing

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7.1 Introduction

Hereditary retinal dystrophies are a common subset of genetic eye diseases causing irreversible visual

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impairment. In most cases, the initial defects mainly affect photoreceptors or retinal pigment epithelium, while, in rare cases, the initial defects may involve early development of vitreous, retina, or choroid. Of these, diseases with initial photoreceptor involvement include retinitis pigmentosa (RP) and its associated diseases, Leber congenital amaurosis (LCA), cone or cone-rod dystrophy (CORD), congenital stationary night blindness, achromatopsia, and blue cone monochromacy (congenital color vision defects will not be discussed here). On the other hand, diseases with predominant impairment of retinal pigment epithelium are composed of vitelliform macular dystrophy, Stargardt macular dystrophy, fundus flavimaculatus, familial dominant drusen, Sorsby macular dystrophy, North Carolina macular dystrophy, butterfly macular dystrophy, etc. Congenital vitreoretinopathies, choroideremia, nanophthalmos, and ocular albinism are examples of developmental disorders involving vitreous, retina, or choroid.

7.2 Clinical and Genetic Heterogeneity of Hereditary Retinal Dystrophies

Hereditary retinal dystrophies are clinically and genetically highly heterogeneous. Most of them can be classified into different entities based on major clinical signs and manifestation. A small port of them may have overlapping phenotypes that might be difficult to be classified into any entity. In rare situation, the phenotype in early stage may represent one disease but that in the later stage may be undifferentiated from others. Variable expression of signs and symptoms can be found in different patients with the same kind of disease or caused by mutations in the same gene, in different affected members from the same family, or even in different eyes of the same patient.

Hereditary retinal dystrophies may be inherited as autosomal dominant, autosomal recessive, or X-linked trait. However, most patients seen in clinic are sporadic cases without a family history. Most of such singleton cases were previously considered to be caused by mutations in recessive genes. Many recent studies have shown that many singleton cases may be caused by de novo mutation in dominant genes. Some of them may be caused by de novo mutation in X-linked genes or transmitted from unrecognized carriers of X-linked mutations. Digenic inheritance, triallelic inheritance, or modified genes have also been proposed to be involved in the genetics of hereditary retinal dystrophies. Complex traits have been demonstrated as the major cause for age-related macular degeneration as well as potential major cause for several other common eye diseases involved in the retina. In this chapter, only Mendelian form or monogenic form of hereditary retinal dystrophies will be discussed further.

Family-based candidate genes analysis and linkage studies have identified a great number of genes responsible for hereditary retinal dystrophies when mutated. Mutations in a number of additional genes have been suggested to cause such diseases based on whole exome sequencing in recent years. So far, mutations in at least 240 genes have been reported to cause hereditary retinal dystrophies, including a small subset of genes for age-related macular degeneration, based on the summarized information from Retinal Information Network (RetNet: https://sph.uth.edu/retnet/home.htm. Accessed on December 31, 2015). A small portion of these genes have been well studied, while genetic analysis of many other genes has been only mentioned in a few reports. In rare cases, some reported mutations in known genes or all reported mutations in a few genes have been revealed to be potentially not causative. Understanding the mutation spectrum as well as their related phenotypes may provide a brief overview of the genetic contribution of known genes in these diseases. In addition, recognizing the importance of family analysis will be very helpful in determining the potential role of a variant and will be critical in clinical mutation detection.

7.3 Mutation Spectrum of Genes for Hereditary Retinal Dystrophies

Mutation detection of those genes responsible for hereditary retinal dystrophies has been mainly relied on analysis of individual gene or a small subset of

genes by using Sanger sequencing in the last two decades. Some of these genes may be frequently analyzed on patients from different ethnic groups with many reports, while many other genes might have been only screened in a small cohort of patients with a few reports. Based on a number of studies from individual gene analysis, it has been suggested that mutations in a few genes are more frequently seen in different populations, while mutations in most of these genes are only responsible for less than 2% of the patients. Systemic analysis of all or most of these genes in the same cohort of patients may provide a more reliable bird view of the mutation spectrum in a given population, but it has become possible until recent years due to the application of whole exome sequencing.

In our lab, several cohorts of patients with common types of hereditary retinal dystrophies, including RP, LCA, and CORD, have been initially analyzed by Sanger sequencing and then analyzed by whole exome sequencing (WES). Whole exome sequencing on genomic DNA of probands with retinitis pigmentosa identified potential pathogenic mutations in known RP genes in 79 of 157 families [1]. Analysis of other genes listed in RetNet identified mutations in additional genes in a small portion of families [2–4]. Taking into account of mutations from WES analysis as well those from previous Sanger sequencing, potential pathogenic mutations have been detected in 110 families with RP in our lab [1–6]. Mutations in the 110 families occurred in 33 of the 240 RetNet genes (Fig. 7.1), in which mutations were more frequently detected in RHO (15/110), USH2A (14/110), RPGR (11/110), CRB1 (8/110), RP2 (6/110), and CHM (6/110) (please note that CYP4V2 mutations were not counted here).

Mutations that were detected in 93 of 159 (58.5%) Chinese families with LCA have been screened by either Sanger sequencing or WES or both (Fig. 7.2) [7–10]. Of these, WES detected mutations in 63 of 108 (58.3%) families. Commonly mutated genes in the 93 families include GUCY2D (17/93), CRB1 (15/93), CEP290 (11/93), RPGRIP1 (8/93), IQCB1 (7/93), CRX (6/93), and ALMS1 (6/93).

Sanger sequencing and/or WES identified mutations in 92 of 162 (56.8%) Chinese families with cone or cone-rod dystrophy (Fig. 7.3) [9, 11–16]. WES alone could detect mutations in 64 of 109 (58.7%) families. The most frequently mutated genes were CNGA3 in which mutations were found in 53 families. The extremely high frequency of CNGA3 mutations in Chinese families with CORD is unexpected since mutation in this gene has not been considered as a common cause of CORD or achromatopsia in Caucasians. It was not listed as the causative gene for CORD before our study. Apart from CNGA3, mutations in several additional genes are frequently seen in Chinese families with CORD, including ABCA4 (6/92), GUCY2D (5/92), ALMS1 (5/92), and CACNA1F (4/92).

In summary, we have identified potential pathogenic mutations in 110 families with RP, 93 families with LCA, and 92 families with CORD. The overall contribution of individual genes for all three types of hereditary retinal dystrophies (Fig. 7.4), i.e., for all the 295 families, is different from that for RP, LCA, or CORD. It is interesting that mutations in several genes, such as CRX, CEP290, and SNRNP200, could be detected in families with RP, CORD, or LCA. In addition, mutational in additional 12 genes was identified in families with two of the three different phenotypes. Mutations in CNGA3, CRB1, GUCY2D, RHO, USH2A, CEP290, ALMS1, RPGR, ABCA4, CRX, and RPGRIP1 has been detected in 53, 23, 22, 15, 15, 13, 11, 11, 10, 10, and 10 families, respectively. This type of relative frequency will be changed again if mutation of these genes in other forms of retinal dystrophies would have been taking into account, such as those in families with early-onset high myopia [17] and Stargardt disease [18]. Further analysis of these genes in all types of hereditary retinal dystrophies may provide a clear overview of mutation frequency in all causative genes.

Apart from overall mutation spectrum for all RetNet genes, it is important to understand the mutation spectrum as well as nature of mutation in individual genes. Mutations in some genes are most frequently occurred in certain exons, such as the ORF 15 mutations of RPGR [19] (Fig. 7.5) and the c.802-8_810del17insGC mutation in

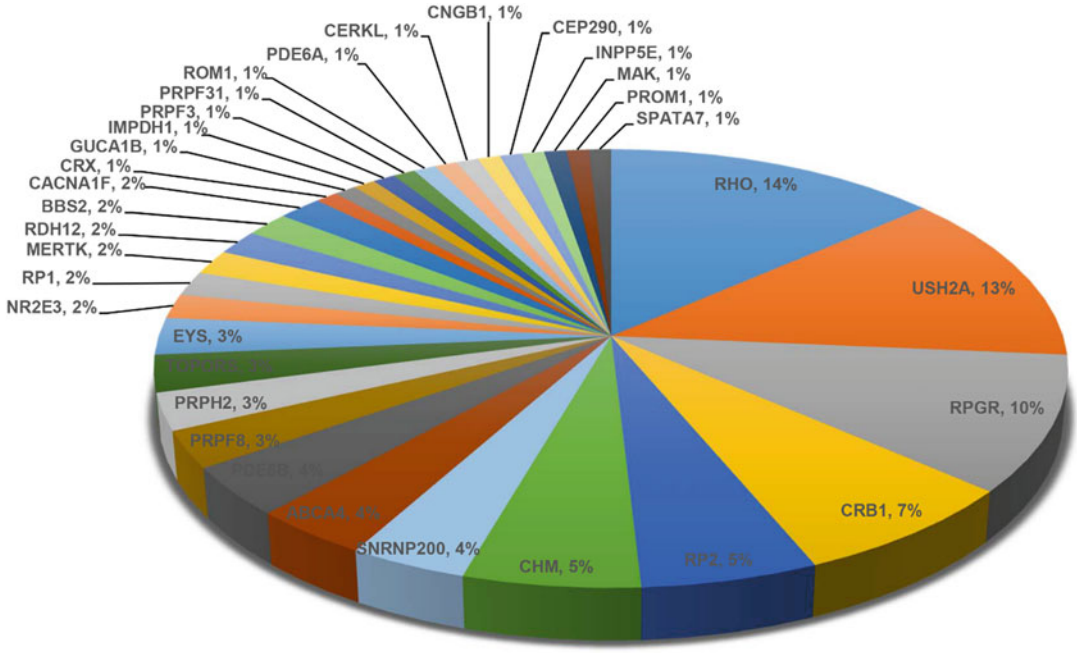

Fig. 7.1 Mutation distribution in RetNet genes of the 110 families with RP and identified mutations. Most of the mutations were identified in known RP genes, while a small fraction was found in genes responsible for other forms of retinopathy

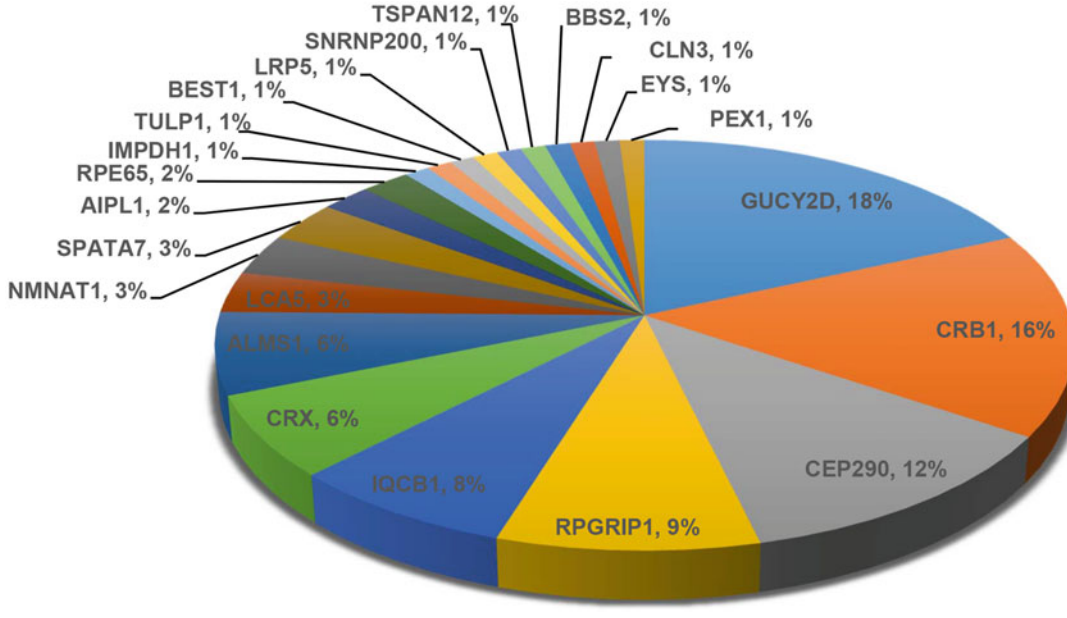

Fig. 7.2 Mutation distribution in RetNet genes of the 93 families with LCA and identified mutations. Most of the mutations were identified in known LCA genes, while a small fraction was found in genes responsible for other forms of retinopathy

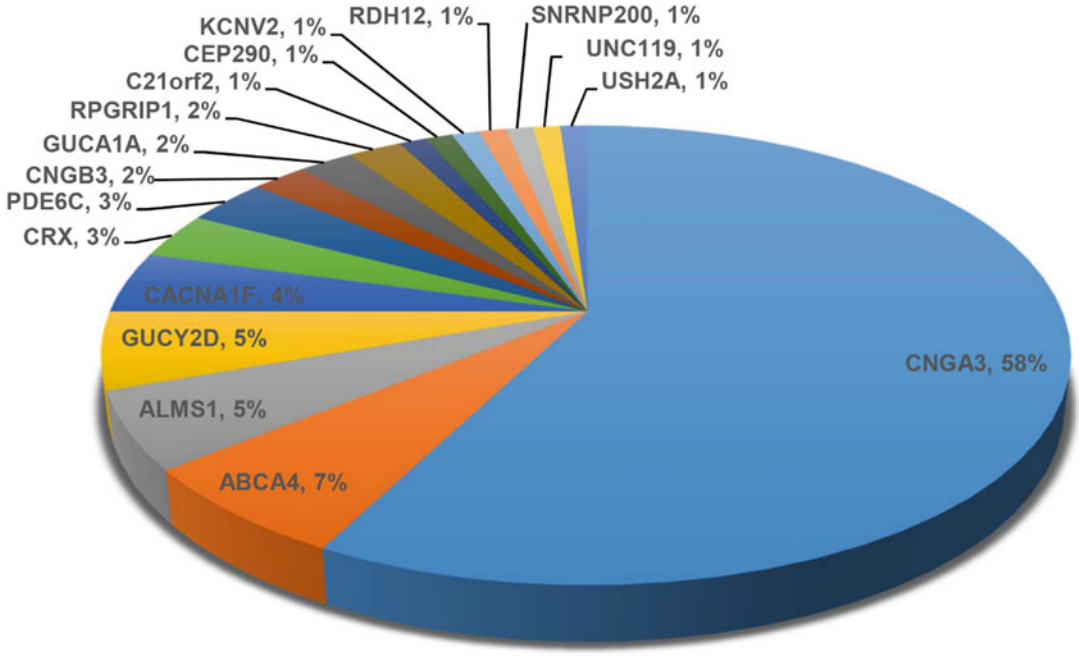

Fig. 7.3 Mutation distribution in RetNet genes of the 92 families with CORD and identified mutations. Most of the mutations were found in CNGA3, while a small fraction of mutation was found in other genes previously known to cause CORD

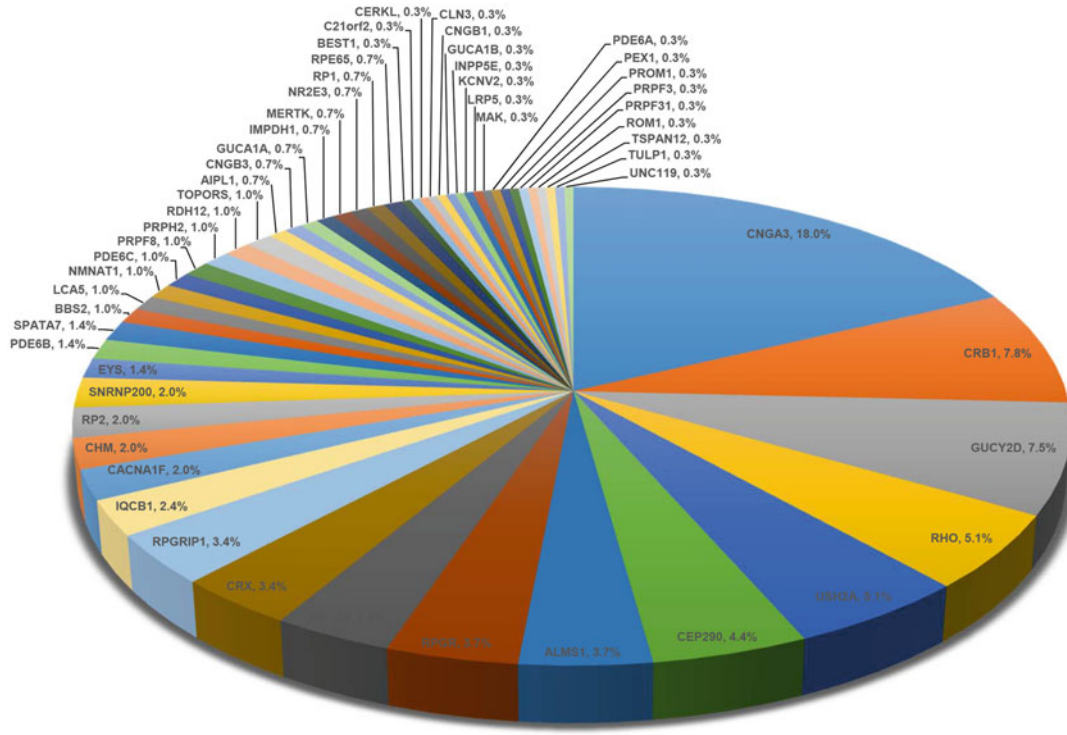

Fig. 7.4 Overall distribution of mutations in 295 families with either RP, or LCA, or CORD. Mutation was detected in only 54 genes in our cohorts although many other genes have been reported to cause these diseases when mutated. It is possible to detect about 95% of mutation for these diseases by analyzing about 40 of the 240 (17%) RetNet genes

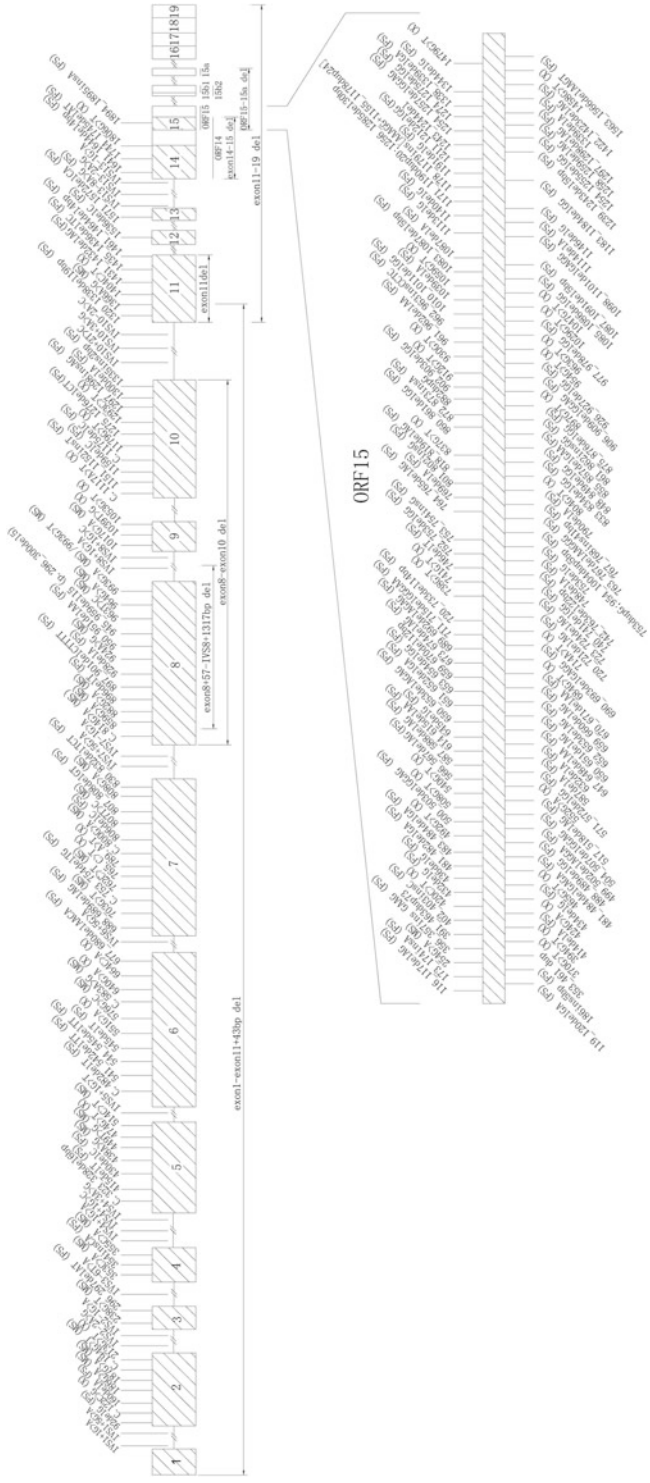

Fig. 7.5 Distribution of part of the previously reported mutation in RPGR, which highlighted mutation hot spot in ORF15

Table 7.1 Spectrum and frequency of CYP4V2 mutations identified in patients with Bietti crystalline corneoretinal dystrophy

No.	Exon	CYP4V2 mutations			No. of allele
		Name	Effect	Type	
1	1	c.130T>A	p.W44R	Missense	1
2	1	c.181G>A	p.G61S	Missense	1
3	1	c.214+1G>A	Exon1del	Splice site	1
4	2	c.215-2A>G	Exon2del	Splice site	1
5	2	c.215-1G>A	Exon2del	Splice site	1
6	2	c.237G>T	p.E79D	Missense	1
7	2	c.253C>T	p.R85C	Missense	1
8	2	c.283G>A	p.G95R	Missense	1
9	3	c.332T>C	p.I111T	Missense	6
10	3	c.335T>G	p.L112X	Nonsense	1
11	3	c.367A>G	p.M123V	Missense	1
12	3	c.400G>T	p.G134X	Nonsense	1
13	4	c.518T>G	p.L173W	Missense	3
14	5	c.655T>C	p.Y219H	Missense	1
15	6	c.732G>A	p.W242x	Nonsense	1
16	6	c.761A>G	p.H254R	Missense	1
17	7	c.802-8_810del17insGC	Exon7del	Indel	128
18	7	C.958c>T	p.R320X	Nonsense	1
19	7	c.971A>T	p.D324V	Missense	1
20	7	c.974C>T	p.T325I	Missense	2
21	8	c.1020G>A	W340X	Nonsense	3
22	8	c.1021T>C	p.S341P	Missense	1
23	8	c.992A>C	p.H331P	Missense	15
24	9	c.1091-2A>G	Exon9del	Splice site	13
25	9	c.1157A>C	p.K386T	Missense	1
26	9	c.1169G>A	p.R390H	Missense	1
27	9	c.1187C>T	p.P396L	Missense	2
28	9	c.1198C>T	p.R400C	Missense	1
29	9	c.1199G>A	p.R400H	Missense	3
30	10	c.1226-6_1235del16	Exon 00del	Deletion	1
31	10	c.1348C>T	Q450X	Nonsense	4
32	10	c.1445C>A	p.S48X	Nonsense	1
33	11	c.1523G>A	p.R508H	Missense	2
34	11	c.1526C>T	p.P509L	Missense	1
Total					204

From Xiao et al. [20]
Spectrun and frequency of CYP4V2 mutations identified in BCD patients

exon 7 of CYP4V2 (Table 7.1) [20]. For several genes associated with autosomal recessive retinopathy, a few particular mutations may cause autosomal dominant retinopathy, such as mutations altered Arg838 of GUCY2D [16] and the R373C mutation in PROM1 [21]. Most mutations in a few genes, such as CRX (Fig. 7.6), are disease causing [13], while only part of the mutations in other genes, like RHO, is causative. For some genes, usually certain type of mutations, such as truncation mutation, is frequently reported to be causative, such as those in ALMS1 [9] and RPGR [22]. Truncation mutations occurred at a different part of a gene, such as RP1, associated with either dominant or recessive RP [23]. Therefore, understanding the

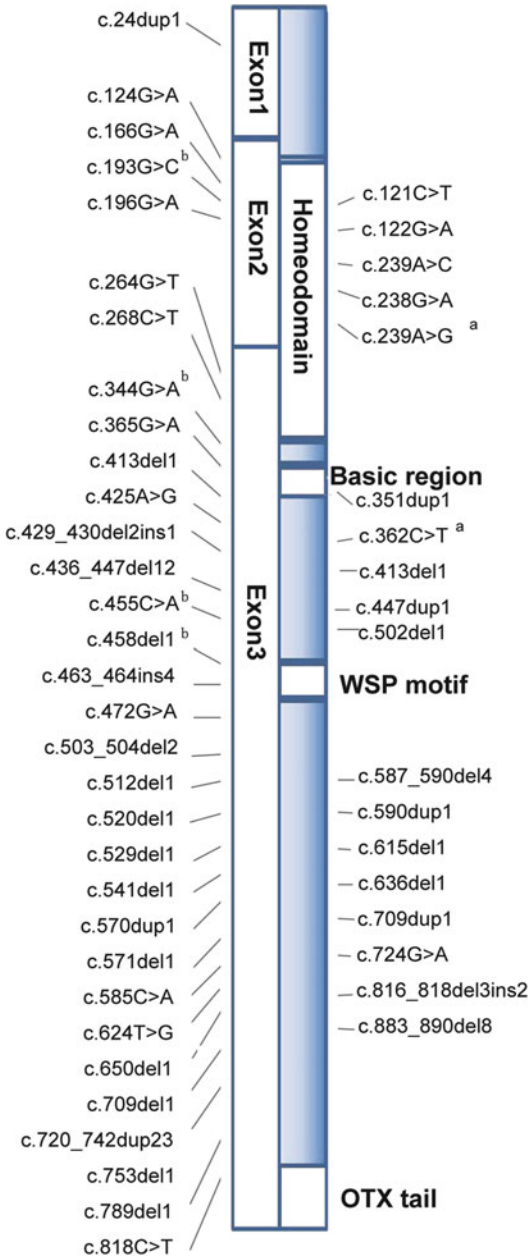

Fig. 7.6 Part of mutations reported in CRX. Mutations listed on the *left side* were found in patients with LCA or RP, while those on the *right side* were from patients with CORD. A small "a" indicated mutations identified in our lab, while a small "b" represented mutations identified in patients with RP (From Huang et al. [13])

complicated association between a mutation and hereditary retinal diseases plays a key role in gene diagnosis as well as in mutation-based drug or gene therapy, which relies on the systemic analysis of gene variants and careful definition of their associated phenotypes in different populations. Segregation analysis in family members of detected variants usually provides a quick and cost-effective way in authenticating its pathogenic role, whether in a proband with or without a family history. Besides, analysis of family members with hereditary retinal dystrophies may provide additional helpful information to further our understanding on these diseases as follows.

7.4 Importance of Family Analysis

No matter large or small, families with hereditary retinal dystrophies are unique resource not only for identifying the molecular genetic basis of related diseases but also for providing novel tool in disclosing the functional role of genes involved in visual pathway.

1. Localization of loci of genes for hereditary retinal dystrophies
   Genome-wide linkage scan or candidate gene-based linkage analysis usually provides firm evidence for localizing the locus of the disease gene in human genome. Otherwise, it would be very hard to find a gene in the huge human genome before the application of next-generation sequencing. A locus located outside of known genes usually indicates that a novel gene for a disease is situated in the linked region. So far, the discovery of most of the genes known to be responsible for hereditary retinal dystrophies is based on investigation of large families with different forms of related diseases (RetNet). The genes at most mapped loci have been finally determined for hereditary retinal dystrophies (RetNet). A series of small families with the same phenotype could also be used to identify the genetic locus, such as the finding of CYP4V2 responsible for Bietti crystalline corneoretinal dystrophy [24, 25].
2. Dominant and recessive inheritance due to mutations in the same gene

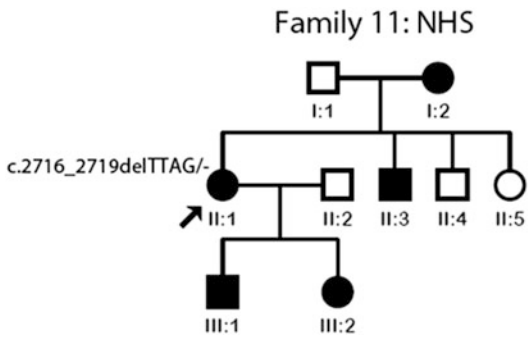

Fig. 7.7 Patients in the family had congenital cataract that was caused by a c.2716_2719delTTAG mutation in the X-linked NHS gene. The pedigree showed a pseudo-dominant inheritance. (From Sun et al. [31])

Mutations in some genes, such as RP1, GUCY2D, PROM1, LRP5, etc., are responsible for autosomal dominant and autosomal recessive retinal dystrophies. Families with characteristic phenotype in all affected members and segregation of mutation with the disease in the families provide unequivocal evidence to demonstrate mutation-disease association with different patterns of inheritance, although different patterns of inheritance-related mutation in the same gene are due to different nature or location of the mutations.

3. Different phenotypes of mutation in one gene
As shown previously, mutations in CRX, CEP290, and SNRNP200 could be detected in families with RP, CORD, or LCA, and those in many other genes were found in two different forms of diseases. A number of studies on families did support the varied expression of the diseases. Phenotypic variations may be due to different nature of the mutation, like those seen in OPN1LW in which unique variants cause both syndromic and nonsyndromic high myopia [26, 27], while unequal crossing-over leading to loss of OPN1LW causes protanopia [28]. Sometimes, the ocular phenotypes may be the only signs or the only noticeable manifestation for several syndromic diseases, such as patients with mutations in the COL2A1, KIF11, and ALMS1 genes [9, 17, 29]. It is also possible that modifiers may play roles in varied phenotypes, especially for those patients with different forms of diseases but with similar types of mutations. Examination at different time points may also contribute to such phenotypic variation since the diseases may look similar at the advance stage. Patients of different ages from the same family provide a unique opportunity to observe the age-related development of the disease, an alternative way for longitude observation.
4. Benign variants, incomplete penetrance, and wrong genes
A few commonly reported variants in a known disease-associated gene may be more likely to be rare benign variation rather than mutation, like the c.3361A>G (p.N1121D) variant reported in LRP5 for familial exudative vitreoretinopathy. These types of rare benign variants will be revealed increasingly at the era of next-generation sequencing. Incomplete penetrance, late onset phenotypes, or atypical and/or varied phenotypes may frequently be observed, which might lead to improper conclusion without great care. Understanding the golden feature of the disease signature would greatly reduce the false incomplete penetrance, i.e., typical disease hallmark can be detected by fluorescein angiography in almost all subjects who carried disease-causing mutations of FEVR-related genes but some of them might be clinically healthy with normal vision and normal-like appearance of the posterior fundus. In rare cases, reported mutation-disease association may not be true, as seen in FSCN2 [30]. We may see a handful of genes which will be finally proved to be wrong-calling causative genes for hereditary retinal diseases in next few years.
5. Pseudo-recessive and pseudo-dominant inheritance
Hidden consanguinity as well as some X-linked diseases may exhibit a pedigree mimicking autosomal dominant inheritance. It is not uncommon to see pseudo-dominant inheritance in families with congenital cataracts caused by NHS mutations (Fig. 7.7)

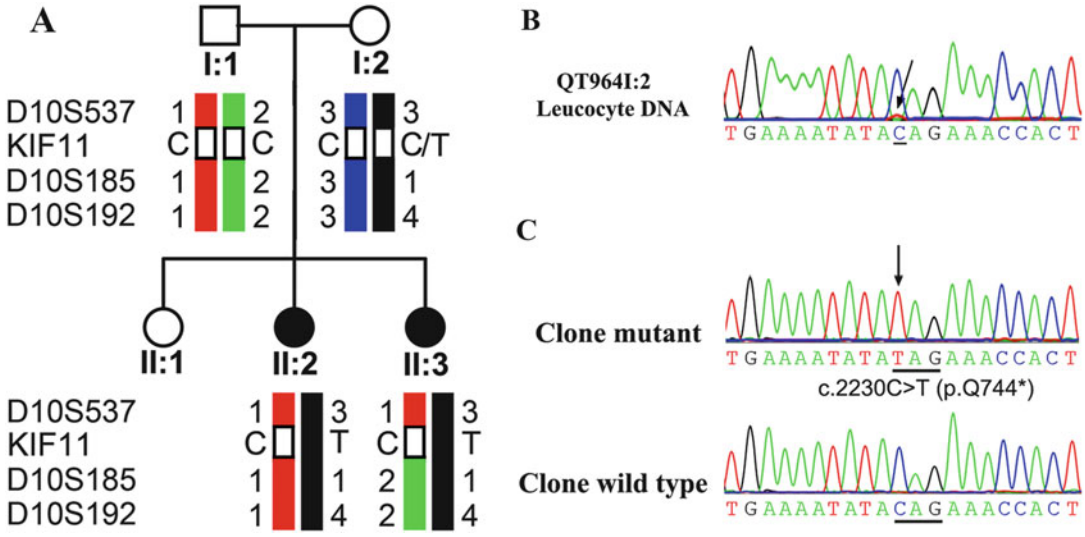

Fig. 7.8 A c.2230C>T mutation in KIF11 was detected in both siblings with FEVR but not in the clinically asymptomatic parents. Genotyping and haplotype analysis showed that the c.2230C>T mutation might be inherited from the mother (**a**). Sequencing chromatography of the mother showed a small variant peak for the mutant allele c.2230C>T (**b**), indicating the existence of mosaicism with a low mutant allele frequency. Based on cloning sequencing of the PCR fragments from the mother's leukocyte DNA, the c.2230C>T was present in about 7% (5/72) of the clones, which confirmed the presence of somatic mosaicism (**c**) (From Hu et al. [29])

[31]. On the contrary, families with parental somatic mosaicism could pretend a pedigree of autosomal recessive pattern of inheritance, as we have seen in a family with KIF11 mutation and familial exudative vitreoretinopathy (Fig. 7.8) [29].

6. The same disease in a family caused by different mutations

   A disease in a family may not necessary be caused by the same mutation. This has been well demonstrated by a two-generation family with Stargardt disease, in which different combination of two of the four different mutations in ABCA4 contributed to multiple macular lesions with a diagnosis of Stargardt disease [32]. We also observed a family with early-onset high myopia, in which different patients had different mutation from RP2 or RPGR. This may cause significant problems in the identification of new genes.

7. Multiple phenotypes caused by mutations in different genes

   In addition to major clinical manifestation, unusual or additional sign might be considered as an expansion of the phenotypes for a gene known to associate with hereditary retinal dystrophies. This is not uncommon in previous studies, as demonstrated by variable phenotypes associated with mutations in PAX6, CACNA1F, RPGR, etc. Sometimes, however, an additional sign may be caused by mutation in another gene, i.e., mutations in two different genes contribute to complicated phenotypes. We have identified a patient with retinitis pigmentosa and Best-like macular dystrophy, who had mutations both in the RP2 and Best1 genes. This type of additional sign is not an expansion of existing phenotypes, neither a new disease nor a new syndrome. Similar situation has also been reported for other disease, such as an atypical form of ataxia with oculoapraxia

type 2 with myoclonus is associated with mutation in SETX and AFG3L2 [33].

7.5 Summary

From the literature and from the clinical practice, usually attention has not been well paid to the unaffected family members of patients with hereditary retinal dystrophies. A few examples in this chapter are offered to highlight the novel value of family study. Indeed, careful clinical and genetic analysis of patients and their family members, either for large families or for singleton cases, could provide important information regarding molecular basis of the disease, pathogenicity of a mutation or even a gene, phenotypic characterization and phenotypic variability, and progress and prognosis. Systemic clinical evaluation of the eye as well as of related organs is extremely important at the age of next-generation sequencing since this may not only help to understand hereditary retinal dystrophies but also to investigate other related but not yet developed abnormalities.

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Whole Genome Sequencing in Patients with Retinitis Pigmentosa

8

Koji M. Nishiguchi

Abstract

Retinitis pigmentosa (RP), the most common form of retinal dystrophy, for which numerous disease-associated genes have been identified, has been studied mainly through the investigation of patients of Caucasian descent. Meanwhile, the genetic background of RP patients in Asia has remained elusive for many years because countries in this region lacked infrastructure and resources to conduct genetic research. However, those circumstances are changing rapidly. The recent economic growth of Asian countries and interest in Asian markets have attracted a large investment in the field of genetics, resulting in a substantial improvement in the research environment. Furthermore, the recent application of next-generation sequencing (NGS) technology has enabled the assessment of numerous candidate genes at an affordable cost, finally providing an opportunity to elucidate the genetics of RP in Asian countries.

To date, a few sequencing platforms based on NGS, including targeted sequencing of a panel of retinal dystrophy genes, whole exome sequencing, and whole genome sequencing (WGS), have been applied to study the genetics of Asian RP. In this chapter, the methodological features of WGS are discussed in comparison with other NGS-based platforms, along with the results following application of WGS for the study of Asian RP patients.

Keywords

Retinitis pigmentosa • Whole genome sequencing • Next-generation sequencing • NEK2 • DFNB31

8.1 Introduction

Identification of the genetic bases of rare inherited retinal diseases (IHD) is becoming an increasingly important step in developing

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effective therapies for the condition. The validity of this concept has been demonstrated with some success for disease gene-guided treatment approaches, including gene therapy [1–3] and retinoid drug administration [4], both targeting a group of patients with autosomal recessive inheritance and defects in a gene encoding an essential member of the visual cycle. The group includes patients with Leber congenial amaurosis caused by genetic defects in *RPE65* gene, encoding the isomerase of the visual cycle [5]. These treatments are specific in terms of their mode of action. Therefore, the treatment efficacy can be assessed reliably by a set of hypothesis-driven clinical tests. Although the effects of treatments have left some room for improvement in various respects, the partial success of these studies has provided acceptable options for these patients and has paved the way for the development of disease gene-guided therapy for intractable IHD. The impacts of the breakthrough in gene replacement therapy have been particularly strong. The concept of supplementing the nonfunctional gene in the diseased retina through subretinal delivery of adeno-associated virus (AAV) is extremely versatile. Therefore, the concept was immediately translated to other disease genes and associated retinal dystrophies for which the same approach can potentially offer therapeutic benefits. Not surprisingly, the studies have sparked the commencement of several clinical trials offering gene replacement therapy worldwide [6]. These include gene supplementation therapy for choroideremia, in which the defective *CHM* gene encoding Rab escort protein 1 was delivered by AAV2 that mainly targets the retinal pigment epithelium [7]. The concept of the gene therapy in this study differs vastly from the earlier ocular gene therapy trials. It has harbored two huge challenges. First, the treatment was aimed to halt the progression of disease rather than restoring vision, the difficulty of which lies in detecting the efficacy of the therapy. Second, a small residual island of healthy ocular tissue underwent therapeutic infection of the virus through an invasive procedure that involves local detachment of the retina, in many cases involving the fovea. This procedure has presented a significant challenge for patients because the effect of the treatment depends on a fine balance between the expected damage caused by iatrogenic retinal detachment and the therapeutic effect of gene supplementation. Nevertheless, the study demonstrated that such a seemingly invasive treatment can be tolerated to a certain degree even in advanced retinal dystrophies, greatly expanding the range of diseases that can benefit from the gene therapy. This includes RP, which shares many cardinal clinical features with choroideremia, especially with respect to the mode of disease progression.

8.2 Retinitis Pigmentosa and Mutation Detection Rate

Retinitis pigmentosa (RP) is a group of hereditary retinal diseases that causes primarily the degeneration of rod photoreceptors and thereby impairs vision in dim light; the disease causes less damage to cone photoreceptors, which are most useful for daylight vision. These different effects are best demonstrated by an electroretinogram (ERG), at least in the early stages of the disease, during which reduction of responses recorded under dark is more pronounced than those recorded after light adaptation. Commonly, ERG responses appear to be disproportionately poorer (smaller and more delayed) than subjective measurements of visual function, such that patients with mild visual field loss and normal visual acuity can show non-detectable ERG responses. Patients typically complain of night blindness from the early stages of the disease. Those symptoms are usually followed by a progressive loss of the peripheral visual field. However, as cone photoreceptor degeneration also ensues with some delay, eventually the entire visual field can be lost, causing complete blindness. Visual acuity might or might not be compromised in the early stages. The fundus of the patients typically shows retinal arteriolar attenuation, scattered intraretinal pigments in a bone spicule configuration, and diffuse abnormalities of the retinal pigment epithelium.

These clinical features can yield a diagnosis of clinically distinct RP. However, when a significant loss of cone function accompanies that of the rods, it is often difficult to distinguish the condition from cone-rod dystrophy (CRD), a clinical entity that is characterized by the preferential loss of cone function over rod function. In other words, RP and CRD can be regarded as a disease of an overlapping spectrum. The limitation of clinical clues to differentiate one retinal dystrophy from another underscores the need for a better diagnostic standard based on the disease pathogenesis, which is best served by ascertaining the genetic causes.

To date, over 200 genes have been assigned as causes of RP and associated disorders (RetNet; http://www.sph.uth.tmc.edu/retnet/home.htm). Application of next-generation sequencing (NGS) to screen numerous RP genes in Caucasian RP patients has revealed mutation in 50–80% of cases [8, 9].

However, the genetic background of RP is also population-specific. In contrast to Caucasians, the mutation detection rate for Asians appears to be generally lower. For example, genetic screening of a panel of RP genes, herein, termed targeted exome sequencing (TES), or whole exome sequencing (WES), in Chinese and Korean patients with RP has led to a mutation detection rate of approximately 50 % [10, 11]. The figure might be even lower among Japanese people. One study with TES of 193 genes comprising mostly genes previously known to be associated with inherited eye diseases in a cohort of 329 Japanese RP patients revealed that only ~36 % were identified with pathogenic mutations [12]. In another study, WES of 30 Japanese RP patients found a mutation detection rate of approximately 26 % [13].

8.3 Whole Genome Sequencing and Comparison of Comprehensive Genetic Analysis Methodologies

The recent evolution of sequencing technologies has expanded our options to tackle extremely heterogenous Mendelian diseases, such as RP, through next-generation sequencing and comprehensive genetic analyses. However, the degree of "comprehensiveness" varies among available approaches, the applications of which are currently limited by financial costs and time and knowledge necessary for analyses.

Mainly, three approaches have been reported for genetic studies of retinal dystrophies. Sequencing of a panel of RP genes through selected amplification processes, TES, is currently the most affordable approach. This approach targets only a small fraction of genes compared to other approaches, which analyze all the annotated genes in the genome. Therefore, sequencing costs can be reduced considerably. However, the degree of complexity of the entire analysis process itself is not extremely different from those of other approaches. Therefore, costs that are not directly associated with sequencing bear a larger weight. However, whole exome sequencing (WES) amplifies and reads all the annotated gene – exons and intron – exon boundaries. Because annotated genes comprise only approximately 2 % of the entire genome, the sequencing cost is still considerably lower than that of whole genome sequencing (WGS) that reads the full genome nonselectively. In WGS, although the sequence data to be obtained are considerably more bulky than data from either TES or WES, selective amplification of a specific region of genome, an extra step, is not required. Therefore, sequencing costs are greatest for such approaches. The lack of artifacts inherent in the amplification process is another obvious advantage of WGS: clearly, WGS allows higher and more even coverage of the genome with greater specificity. Moreover, WGS facilitates investigation of various genetic elements located in the noncoding genome including noncoding RNAs, promoters, cryptic exons, and intronic variants that are known to cause disease but which are unapproachable with other platforms. Such investigations can include those of structural changes such as large inversions, insertions, and deletions with a boundary lying deep in introns. Furthermore, comprehensive transcriptome analyses are uncovering many new tissue-specific transcripts that fall outside of well-characterized reference

cDNA. Consequently, identification of pathogenic mutations affecting newly identified tissue-specific isoforms might be possible only after retrospectively integrating the new transcriptome data obtained from the retina. This is only possible in the WGS platform.

Currently, a few NGS platforms are commercially available. Some differences confound the data interpretation [14]. With regard to WGS, most commonly used platforms are those provided by Illumina and Complete Genomics (CG). Performance comparisons of these methods have been conducted and reported [15]. Important technological differences include the length of the paired-end reads used (101-bp in Illumina and 35-bp in CG). The study revealed that, although both platforms showed similarly high genomic coverage (>95 % was covered by 17 or more reads) and a high concordance rate (88.1 % comprising 3295, 023 SNVs) of single nucleotide variation (SNV) calls, a significant number of SNV calls specific to each platform were also obtained. Specifically, 345,100 were specific to Illumina (10.5 % of total Illumina SNVs), and 99,578 were CG specific (3.0 % of total CG SNVs). The difference between these two platforms arises from the difference in the specificity of the variation calls. Sanger sequencing of randomly selected platform-specific SNVs revealed a high false-positive rate with Illumina (86.7 %) compared to CG (5.6 %). Meanwhile, CG showed less-uniform coverage than Illumina, which demonstrates that CG requires more numerous reads to secure sequence data with a similar level of reliability.

Somewhat disappointing results were obtained for calling insertions and deletions (indels) in both platforms. Although 26.5 % of indels were detected in both platforms, 25.4 % and 48.1 % of identified indels were, respectively, platform-specific to CG and Illumina. Although 54.8 % of platform-specific indels failed to amplify, Sanger sequencing indicated most of the amplified indels as real. Therefore, the sensitivity of detecting indels is low in both platforms. Further improvement is necessary from this perspective.

8.4 Direct Comparison of Whole Genome Sequencing Data of Asians and Non-Asians

To date, only one report of the literature describes a study that has applied WGS to study the genetics of RP using a CG sequencing platform [16]. This study comprised eight Japanese and eight North Americans of diverse ethnic origin (six mixed European, one Haitian, and one Hispanic), all with clinical features consistent with typical non-syndromic RP and a family history suggestive of autosomal recessive inheritance. The average statistic parameters of the WGS from 16 RP patients included gross mapping yield of 200.8 Gb with average coverage of 66.1 per base, called genomic fraction of 96.8 %, and total detected SNV of 3,865,180 (Table 8.1). Because the deposition and accumulation of DNA variant data of Asians compared to those of non-Asians are expected to be notably small from a historical perspective, the first question was whether the flooding variant calls expected in Asian WGS would obscure the search for pathogenic mutations. However, to our surprise, the total number of detected variations was not different between Asians and non-Asians. In fact, the average number of detected variations was even higher in non-Asians (3,972,326 variants) than in Japanese people (3,758,035 variants). Accordingly, the number of novel variants not registered in dbSNP was also higher in non-Asian patients (329,608 variants) than in Asian patients (272,551 variants). These comparative data ensure that variant calling in Asians might not necessarily be more complicated than that of non-Asians.

8.5 Pathogenic Mutation Search in Asian and Non-Asian RP Patients Using Whole Genome Sequencing

To classify the overwhelming number of variants identified through WGS and to search for

Table 8.1 Comparison of whole genome sequencing parameters of Asians and non-Asians

	Americans average	Japanese average	Total average
Gross mapping yield (Gb)	199.4	202.3	200.8
Average coverage (per base)	65.5	66.8	66.1
Fraction of genome covered more than 30×	0.89	0.86	0.875
Called genomic fraction	0.970	0.966	0.968
Total variations	3,972,326	3,758,035	3,865,180
Total novel variations	329,608	272,551	301,079
Total novel rate	0.0814	0.0725	0.0769
Variations not in mRNA	3,950,758	3,737,505	3,844,131
Variations in mRNA	21,569	20,530	21,049
Synonymous	10,499	9948	10,224
Non-synonymous	11,069	10,582	10,826
Missense	10,068 (91.0%)	9549 (90.2%)	9808 (90.6%)
Nonsense	97 (0.9%)	96 (0.1%)	96 (0.9%)
Nonstop	18 (0.2%)	12 (0.1%)	15 (0.1%)
Misstart	19 (0.2%)	29 (0.3%)	24 (0.2%)
Frameshift	278 (2.5%)	272 (2.6%)	275 (2.5%)
Frame preserving	538 (4.9%)	532 (5.0%)	535 (4.9%)
Splice site	52 (0.5%)	92 (0.9%)	72 (0.7%)

Unless stated otherwise, numbers refer to "vars," defined as unique variations within the genome, regardless of their allelic information, so that both homozygous and heterozygous variants are considered a single var

pathogenic mutations, a two-step analytical pipeline based mostly on PERL script was constructed to minimize the data handling complexity. In the first step, variants in 64 known RP-related genes were analyzed. For cases not identified with a pair of convincing pathogenic mutation, the possibility of finding mutations in a novel disease gene was explored in the second step. The step comprised a workflow specifically tailored to select candidate variants based efficiently on the extracted profiles of mutations identified in the known RP genes in the first step.

Main results of the WGS in Asian and non-Asian RP patients are presented in Table 8.2. Nine out of 16 patients comprising four Asians and five non-Asians were identified with a pair of mutations to account for their autosomal recessive mode of inheritance. Mutations were identified in eight different genes, six of which were reported previously as causes of non-syndromic RP.

A homozygous nonsense mutation (p. Q54X) was identified in *DFNB31* gene in an elderly Japanese woman with a parental history of consanguinity and with no history of hearing loss (Patient ID: R18). Homozygosity mapping confirmed the mutations to lie in the genomic region predicted to be identical by descent. This gene had been associated previously with Usher syndrome type II [17], a syndromic form of RP accompanied by a hearing loss, but not with a typical non-syndromic RP.

Another gene, *NEK2*, which was never associated with retinal dystrophy, was identified with a homozygous frameshift variant (p. L206 fs, c.617_624delTGTATGAGinsA) in a Japanese woman also with a parental history of consanguinity (Patient ID: R19). Actually, R19 was one of the 8 RP patients left unsolved after the initial screening of 64 known RP genes. It was subsequently used for a search for mutations in an as yet unidentified gene. The variant was found within a highly homologous genomic stretch (19.6 Mb) of chromosome 1q32 that was judged by homozygosity mapping to be identical by descent. The variant would result in a premature termination of the transcribed mRNA, which would probably be degraded through nonsense-mediated decay producing no protein product. To verify that the identified variants were indeed associated with RP phenotype, a functional assessment was conducted in zebra fish. The

Table 8.2 Summary of genetic analysis of 16 patients with RP

Patient ID	Sex	Cons.	Ethnicity	Gene	Protein change	DNA change	State	Solved/ unsolved
003-019	F	N	Mixed European	*USH2A*	p.C3294W	c.9882C>G	het	Solved
				USH2A		del Ex27	het	
003-050	M	N	Mixed European					Unsolved
003-175	F	N	Mixed European					Unsolved
003-197	F	N	Haitian	*RDH12*	p.R84X	c.250C>T	het	Solved
					p.G76R	c.226G>T	het	
099-046	F	N	Hispanic	*CNGB1*	p.C632X	c.1896C>A	het	Solved
				CNGB1	p.G1050 fs	c.3150delG	het	
218-304	M	N	Mixed European	*CERKL*	p.T260 fs	c.780delT	het	Solved
				CERKL	p.K200X	c.598A>T	het	
219-004	M	N	Mixed European	*CERKL*	p.R257X	c.769C>T	het	Solved
				CERKL	p.L140 fs	c.420delT	het	
218-237	F	N	Mixed European					Unsolved
R4	F	N	Japanese					Unsolved
R8	M	N	Japanese					Unsolved
R9	F	N	Japanese	*EYS*	p.L1723 fs	c.5168_5169insT	het	Solved
				EYS	unknown	invert-dup Ex23-29	het	
R14	F	Y	Japanese	*PDE6B*	unknown	IVS11+1G>C	hom	Solved
R15	F	Y	Japanese					Unsolved
R16	F	Y	Japanese					Unsolved
R18	F	Y	Japanese	*DFNB31*	p.Q54X	c.160C>T	hom	Solved
R19	F	Y	Japanese	*NEK2*	p.L206 fs	c.617_624delTGTA TGAGinsA	hom	Solved

Abbreviations: *F* female, *M* male, *N* no, *Y* yes, *Cons.* reported parental consanguinity, *hom* homozygous, *het* heterozygous

experiment revealed that morpholino-mediated knockdown of the *nek2* mRNA caused a photoreceptor loss which was rescued phenotypically when knockdown morpholino was administered together with nek2 mRNA.

8.6 Intronic Pathogenic Mutations Detected by Whole Genome Sequencing

Aside from the observation that WGS is useful as a tool for genetic diagnosis in both Asian and non-Asian people, the results imply that Asian patients may be enriched with unidentified RP genes or unreported phenotype-genotype correlations. However, such genetic analysis can be performed also with WES.

The salient benefit of WGS over WES in the genetic diagnosis of RP lies in the discovery of two large structural variants that were unidentifiable with other NGS platforms. Coverage-based analysis of copy number variants in WES has been attempted with little success. Limitations of this approach include the uneven coverage yield across the genomic regions captured with different probes and the inability of identifying the junction of the structural variants that are usually located deep in noncoding regions composing approximately 98 % of the entire genome. Furthermore, without

obtaining junction information, even simple genomic inversions cannot be detected.

A large deletion that completely excised exon 27 of *USH2A* gene was identified in an RP patient (Patient ID: 003-019) in combination with a pathogenic p. C3294W mutation (Fig. 8.1). The exact boundaries located deep in the introns flanking exon 27 were identified unambiguously. Another complex structural variant that results in chromosomal inversion and duplication affecting exons 23–29 of *EYS* gene, a gene identified as the most common cause of autosomal recessive RP among Japanese [18], was found together with a clearly deleterious mutation (p.1723fs) in a Japanese RP patient (Patient ID: R9; Fig. 8.2). Again, all boundaries located in the midst of noncoding regions were identified, which facilitated detection of the exact structure of the complex mutation.

The finding that two out of the nine solved cases (22.2 %) had a pathogenic mutation in the noncoding regions that would be defined only by WGS is intriguing in the context of genetic diagnosis. However, it is possible that the importance of analysis of the intronic sequences might be a gross underestimate because only 7 out of 16 genomes were informative for structural variant analysis. Unfortunately, most of the DNAs analyzed after many years of storage lacked reliable mate-pair information that is necessary for detection of junctions. They were therefore excluded from structural variant analysis.

A comprehensive search for an association between RP and noncoding RNA was also conducted, without a fruitful discovery.

8.7 Future of Genetic Analysis of Asian RP Patients

Currently, WGS costs a few times as much as TES or WES. Although WGS outperforms WES and other approaches in terms of the final sequencing output, application of a WES or TES approach is gaining in popularity. However, the landscape is changing constantly in the field of genetic testing. The sequencing cost, which is a main limiting factor when choosing an analysis platform, is falling and is expected to continue falling. Considering that the sequencing cost comprises more than 90 % of WGS, it is feasible to assume that a decline in WGS cost will follow. The main cost of WES is associated with the process of capture and/or PCR amplification, which also appears to be decreasing but at a slower rate [19].

In the context of diagnostic application, it is possible that the process of Sanger sequencing required to confirm the results of NGS might bear an increasing weight in the coming years because there has been only little technical advancement. The cost and the labor associated seem to have nearly stopped changing. Therefore, the four to five times higher false rate inherent in the extra

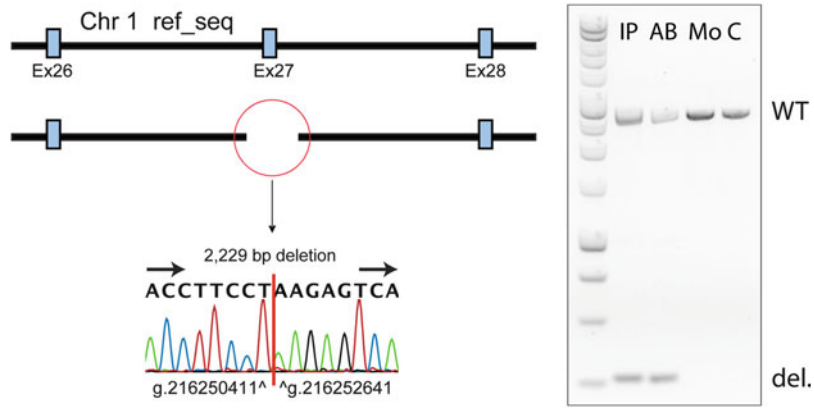

Fig. 8.1 Sequence of the intronic junction resulting in heterozygous USH2A 2229-bp deletion in patient 003-019. Electrophoresis image of the PCR fragments showing a smaller fragment carrying the deletion in the index patient (IP) and her affected brother (AB) but not in her mother (Mo) or a control DNA (C). *del* deleted allele; *WT* wild-type allele

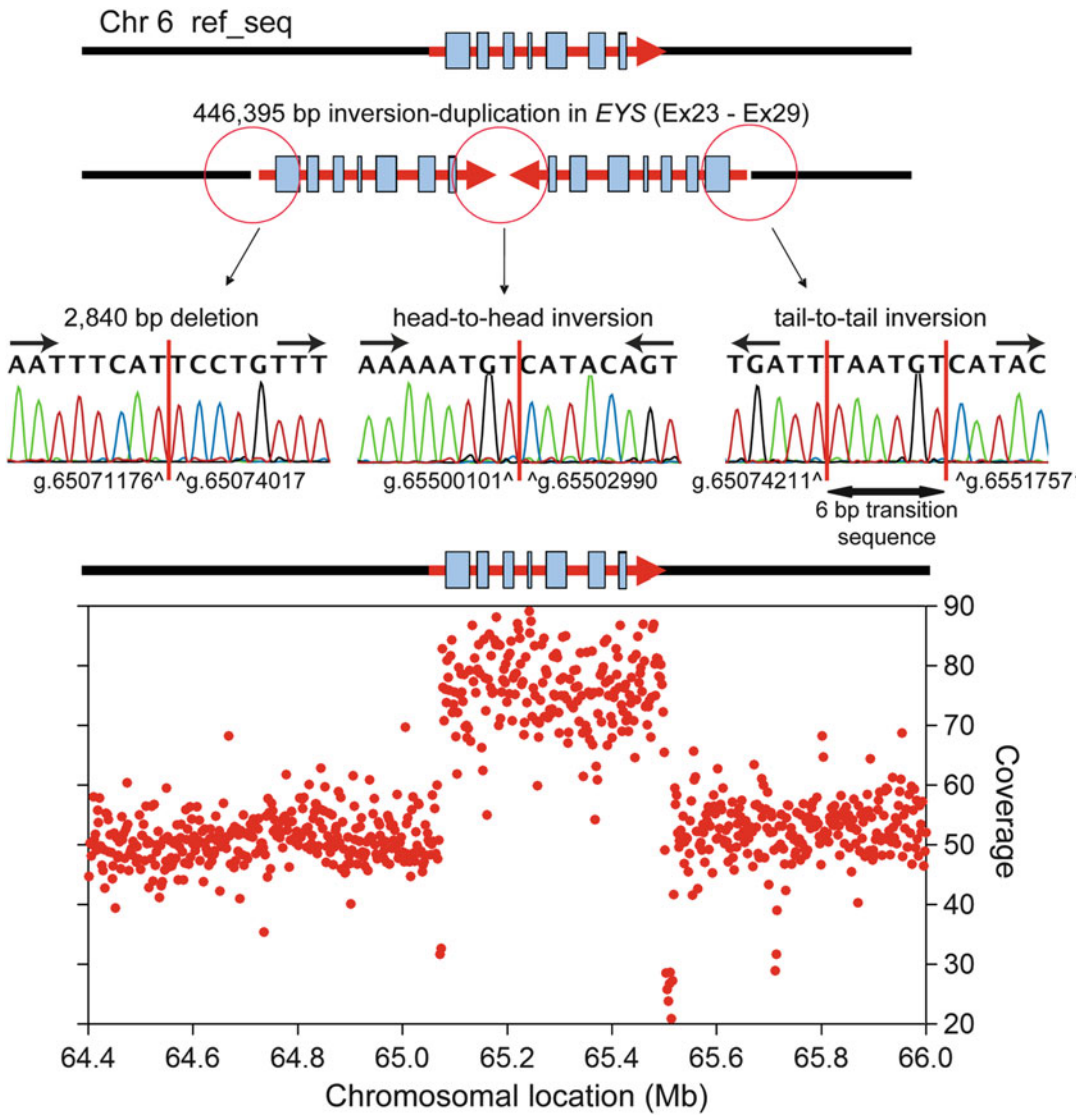

Fig. 8.2 Schematic representation and DNA sequence of the intronic junctions characterizing the chromosomal rearrangement involving the *EYS* gene detected in patient R9 (*upper panel*). Integration of the information obtained by Sanger DNA sequencing and WGS coverage of the region enables identification of an inverted duplication encompassing exons 23–29 (*lower panel*)

capture and amplification step of WES approach, which translates directly into an increased number of candidate mutations to be verified by Sanger sequencing, might impede the overall reduction of the costs of genetic analysis [19].

Lastly, even after applying comprehensive analyses such as WGS or WES, evidence suggests that a large share of Asian RP patients might remain genetically solved [10–13, 20]. Therefore, a limitation is apparent for the conventional strategy of looking only into well-annotated genomic regions, including exons within the framework of simple Mendelian inheritance, to decipher the causes of Asian RP. Results of this study show that WGS facilitates unrestricted investigation of various nonconventional genetic elements including non-coding RNAs, promoters, cryptic exons, and

intronic elements that are reported to cause disease but which are often ignored. Actually, WGS can be regarded as the key NGS platform to unravel the current mysteries related to Asian RP genetics.

Compliance with Ethical Requirements Koji M. Nishiguchi has no conflict of interest in relation to this study.

Human and animal studies were performed after approvals were obtained from the relevant review boards and adhered to the tenets of the Declaration of Helsinki.

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Retinitis Pigmentosa in Korean Patients

9

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Keywords

Retinitis pigmentosa • Cystoid macular edema • Cataract • Rhodopsin • *EYS* • *RP1* • *RP2* • *RPGR*

Retinitis pigmentosa (RP) is a serious disease characterized by progressive degeneration of the retina and usually ends up with bilateral blindness. RP is the most common inherited retinal disease. Recent advance in genetic research has greatly improved our knowledge about RP. Since the first identification of causal gene rhodopsin, more than 60 causal genes and 3000 disease-causing mutations have been reported [1]. And imaging technology such as a high-resolution spectral domain optical coherence tomography (OCT) revealed new aspects of RP [2]. In RP patients, the vision-specific quality of life cannot be explained only by visual acuity or field, and both of them are related to the visual function [3].

Typical nonsyndromic RP prevalence is approximately 1:3000–1:5000 worldwide. The prevalence was 1:5200 in Maine, United States, and birth incidence was calculated to be 1:3500 [4]. Relatively lower prevalence of 1:7000 was reported in Switzerland [5]. The registration for RP revealed the prevalence of 1:3943 in Denmark [6]. The prevalence in Southern China was 1:4000 [7]. Excluding epidemiologic study obtained from small-sized cohort, the prevalence of RP looks similar among the ethnicities. Although the prevalence data was not yet reported in Korea, we can cautiously propose it might not much differ from wordwide prevalence of 1:4000. However, RP seems to be underestimated in Korea, considering that RP accounted for 9.6% of low-vision patients in National Medical Center in Korea [8]. Prevalence survey for syndromic RP is relatively scarce. The prevalence of Usher syndrome is estimated to be 1:6000 [9], and the prevalence of Bardet-Biedl syndrome varies from 1:25,000 to 1:160,000 [10].

9.1 Inheritance Pattern

RP is inherited as an autosomal-dominant (AD, about 30–40% of cases), autosomal-recessive (AR, 50–60%), or X-linked (5–15%) trait if sporadic cases are assumed to be AR [11]. The proportions of AD, AR, X-linked recessive, and simplex cases were reported to be 11%, 33.1%, 7.7%, and 48.3%, respectively, in China [12]. In a study of Japanese RP patients, AD, AR, X-linked recessive, and simplex cases were 16.9%, 25.2%, 1.6%, and 56.3%, respectively

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[13]. Population-based study for RP inheritance pattern has not been performed in Korea yet. However, in a small cohort study including 302 Korean RP patients, the most common inheritance pattern was sporadic (182 patients, 60.3%), followed by ARRP (55 patients, 18.2%), ADRP (38 patients, 12.6%), and an unknown pattern (27 patients, 8.9%) [14]. Other study including 750 Korean RP patients found that the proportion of inheritance pattern was ADRP 11.7% (89 patients), ARRP 13.4% (102 patients), XLRP 4.1% (31 patients), sporadic 56.9% (431 patients), and ambiguous 13.7% (104 patients) (unpublished data). The big picture is thought to be not so different among the East Asian countries.

9.2 Macular Abnormalities

Macular abnormalities are sometimes accompanied by RP, which include cystoid macular edema (CME) and epiretinal membrane. Recently, analysis for macular abnormality has become more accurate with the advent of high-resolution OCT. Among 1161 Italian RP patients, macular abnormality was detected in 524 patients (45.1%). Most abundant abnormality was CME, observed in 237 eyes (20.4%), followed by epiretinal membrane (ERM) in 181 eyes (15.6%). However, Hagiwara et al. revealed that only 46 eyes (7.4%) have macular abnormality in 622 eyes of 323 RP patients using OCT [15]. In that study, CME was detected in 34 eyes (5.5%), and ERM and vitreomacular traction (VMT) were detected in four eyes (0.6%) and five eyes (0.8%), respectively. Makiyama et al. detected CME in 119 eyes (22.5%) out of 529 eyes of 275 patients [16], and they did not found any difference in the incidence of CME among the inheritance patterns. However, cystoid space was associated with the presence of ERM or VMT. Mentioning the Korean RP patients, 46 eyes of 30 patients (20.9%) had CME among 220 eyes in 128 patients [17]. However, in another study in Koreans, CME and ERM were detected in only three eyes (0.8%) and two eyes (0.5%) out of 365 patients [18]. Extremely low prevalence of macular abnormality in the study could be explained by that macular abnormality was only based on funduscopic finding. For a similar reason, low spatial resolution of time domain OCT could have made the relatively small detection rate of macular abnormality in Hagiwara study. Different definitions and different detection method for macular abnormality among the studies seem to be associated with this discrepancy. Nonetheless, most abundant macular abnormality was consistently CME. Various mechanisms, such as anti-retinal antibody, vitreous traction, retinal pigment epithelium dysfunction, and Müller cell dysfunction, have been suggested as the pathophysiology of CME in RP, but none of these are not proven yet. Therefore, it is still unclear whether the various proportion of macular abnormality is associated with the difference in genetic background.

9.3 Cataract

One of the common complications of RP is lens opacities, especially posterior capsular opacity. Pruette reported that lens opacity or pseudophakia was observed in 46.4% of 384 eyes in American RP patients [19]. Among lens opacities, 80% was posterior polar cataract, 10% was nuclear sclerosis, and 20% was both nuclear sclerosis and posterior polar opacity. Although older age group showed higher prevalence of lens opacities, 10.3% of patients younger than 20 showed cataract. In the study, lens opacity was more prevalent in AD inheritance group. Fishman et al. observed posterior subcapsular cataract (PSC) in 53% out of 338 American RP patients whose average age was 38.7 years [20]. PSC was more prevalent in X-linked group, while it was relatively uncommon in AD group. Berson et al. also reported that lens opacities in RP were most prevalent in X-linked inheritance as 72% [21]. Among 365 Korean RP patients, lens opacity was detected in 47.9%, and PSC was most common accounting for 25.8% of total patients. Average age was 38.0 years old, and there was no difference among the inheritance

patterns unlike Pruette et al. and Fishman et al. study. [18] However, population-based epidemiologic study performed in Korea reported the weighted prevalence of cataract in ordinary adult population as 23.5% and PSC as 3.8% [22]. Because of lacking in detailed age data in population-based study, comparison of age-corrected prevalence of cataract and PSC with Korean RP study [15] was not available, but PSC prevalence seems to be high in RP patients.

9.4 Refractive Errors

Refractive errors are frequently reported in inherited retinal dystrophy. Among them, RP has been consistently regarded as associated with myopia. Sieving et al. observed that mean spherical equivalent (S.E.) of RP was −1.86 diopters, which was significantly more myopic compared to that of 2066 normal population by −2.93 diopters [23]. However, this overall S.E. of RP was not so different from recent S.E. data from general population in North America [24]. When dividing RP patients of Sieving et al. into inheritance pattern, spherical mean of X-linked RP was −5.51 diopters, and this was significantly more myopic than −1.2 diopters of non-X-linked RP. Fishman et al. also reported that S.E was greater than −3.00 diopters in 68% of X-linked RP patients [25]. Moreover, higher proportion of myopic refractive errors were consistently observed in X-linked RP resulted from *RP2* and *RPGR* mutations [26, 27]. In addition to X-linked RP, AR inheritance pattern patients having *RP1* mutation also show high myopic refractive errors [28]. In this study, ARRP having *RP2* mutations had myopia ranging from −3.8 to −4.0 diopters, and it was not statistically different from S.E. of X-linked RP patients. Possible explanation for the relation of myopia to *RP2*, *RPGR*, and *RP1* mutation with AR inheritance has not yet been proposed. In Koreans, 77% of 294 RP patients were myopia and mean S.E. was −2.97 diopter [18]. Unlike previous reports, no difference was observed according to the inheritance patterns. In population-based study, prevalence of myopia in Korea was 48.1% and high myopia was 4.0% over the age of 20 [29]. Considering the high prevalence of myopia in Koreans, refractive errors of Korean RP patients should be further studied. Among the patients whose causal variant was revealed, 10 had *RP1* mutations and 11 had *RP2* mutation including affected female heterozygote (unpublished data). All patients were AD and all three patients having refractive data showed myopia of S.E. −2.75 to −7.00 diopter. For *RP2* mutations, five patients have detailed refractive data and showed moderate myopia with mean S.E. of −3.0 diopters. No *RPGR* mutation was noted in Korean patients.

9.5 Genetic Profile of Retinitis Pigmentosa in Koreans

We used massively parallel sequencing for 53 targeted RP gene in 62 nonsyndromic Korean patients. Causal variants were confirmed using Sanger sequencing. Possible causal variants were identified in half of patients. Novel variants were 65%. Although overall distribution of frequently detected RP genes was similar to previous studies performed in other ethnicities, there were some differences in frequency. Rhodopsin mutation was lower and *EYS* mutation was higher in Korean population compared to European population, while recent study with Asians showed a similar proportion including rhodopsin and *EYS* mutation. In X-linked RP, *RPGR* gene is reported to be accounting for over 70% of causative variants. However, *RPGR* gene variants were not detected in Korean cases (Table 9.1 and 9.2).

9.5.1 Rhodopsin Gene

Rho coding rhodopsin is located in 3q22.1 and is inherited as AD pattern. It was firstly found as a causal gene of RP and most frequently found ADRP gene as much as 16–28% [11, 34–36]. Sanger sequencing screening for 302 Korean patients revealed that six (2.0%) were confirmed of Rho mutations [14]. This overall proportion of

Table 9.1 Comparison of gene mutation frequency

Author	Hartong [11]	Eisenberg [30]	Xu [31]	Oishi [32]	Our study [33]
Nationality		Unites States	Chinese	Japanese	Korean
ADRP (100%)					
Rho	25.0%	29%	18.70%	27.0%	20%
PRPF31	5.0%	36%	3.10%	11.6%	30%
RP1	5.5%				20%
PRPF3	4.0%		3.10%		
PRPH2	2.5%	7%	6.30%	15.3%	10%
TOPORS		14%	6.30%	3.7%	10%
SNRNP200			9.40%	11.6%	
PRPF8	2%		6.30%		
CRX	1%		3.10%	7.9%	
RDH12				7.9%	
KLHL7					10%
ARRP (100%)					
USHA2A	20%		34.60%	22.8%	20%
EYS		9.4%	7.70%		40%
RP1	<2.5%	11.3%			
PDE6B	11.2%	7.6%	11.60%	12.5%	40%
PDE6A	8.7%		3.80%		
ABCA4	13.7%	7.6%	11.60%		
TULP1	2.5%	7.6%			
CNGB1	10.0%		3.80%		
MERTK	1.0%		3.80%	6.2%	
RP1L1				14.5%	
CNGA1				6.2%	
MAK				6.2%	
XLRP (100%)					
RPGR	70–80%		57.0%	100.0%	
RP2	7–10%		43.0%		100%

Table 9.2 Causative variants identified in next-generation sequencing analysis in Korean RP patients

Gene	Familial case	Sporadic case	Frequency
PRPF31	2	1	17.6%
EYS	0	2	11.8%
PDE6B	0	2	11.8%
RHO	2	0	11.8%
RP1	2	0	11.8%
RP2	2	0	11.8%
KLHL7	1	0	5.9%
PRPH2	0	1	5.9%
TOPORS	1	0	5.9%
USH2A	0	1	5.9%
Total	10	7	100.0%

Rho in RP was much smaller than Caucasian, which is estimated to be 8–10%, but comparable to 2.0–5.6% of Chinese and 2.0–5.9% of Japanese [37–40]. When confined to ADRP, Rho mutation proportion of 10.5% (4 of 38) in Korean ADRP was similar to 8–15% from Chinese reports [39]. Distribution of Rho gene mutation as RP causal gene in Korea is smaller than European ethnicity but comparable to East Asians. Isolated cases of Rho mutation have been rarely reported. However, Li et al. noted Rho mutation in 5.5% of isolated cases in Chinese [39]. We observed Rho mutation as causal variants in 1.1% (2 of 182) of isolated cases.

Rhodopsin is the light-absorbing photopigment and located in the disc membrane of rod outer segment [41]. It is G protein-coupled receptor family and composed of opsin molecule covalently bound to 11-cis-retinal [42]. Light absorption catalyzes the photoisomerization of

the 11-cis-retinal to all trans-retinal. After that, conformational change leads to metarhodopsin II and activates transducin. Following phototransduction makes neural signal [43]. Although P23H is the most frequent RHO mutation as much as 40% in the US, it has not been reported in Asians including Koreans [34]. In this case protein folding is disrupted and deposited at rough endoplasmic reticulum. Generally, mutation located in intradiscal area or N-terminal affects less severe phenotype than the mutation located in the cytoplasmic region or c-tail [43]. A total of six Rho mutation were found in Koreans, and five (p.T17 M, p.D190N, p.R135W, p. P347L, and p.Y178C) were previously reported, while p.A298D was novel. In our cases, p.P347L also shows severe clinical features. In a family with this mutation, a 44-year-old woman revealed severe foveal atrophy on OCT, while her four children in their teens showed bilateral CME and intact outer retina and photoreceptor ellipsoid zone only around macular region [44]. Aleman et al. reported that the outer nuclear layer was nearly extinguished and the inner nuclear layer was relatively thickened in certain types of mutation including R135W and P347L [45]. Our familial case (p.P347L) also showed this feature, while patients having p.T17 M, p. Y178C and p.D190N mutations located in N-terminal and intradiscal region presented milder clinical form than p.P347L. Useful central vision was preserved until 40s (Fig. 9.1) (Table 9.3) [14].

9.5.2 *PRPF31* and *PRPF3* Gene

PRPF31 is one of the six pre-mRNA splicing factors including PRPF3, PRPF6, PRPF8, SNRNP200, and RP9. Although it is ubiquitously expressed protein essential for general cell metabolism, mutation of PRPF31 only affects the eye without any syndrome. This is a similar phenotype pattern to other pre-mRNA splicing factors. This feature can be partly explained by that these mutations cause inefficient splicing and the retina has particularly high splicing activity compared to other tissues [46]. PRPF31 is a cause of ADRP and the majority of reported causal mutations were truncating mutations. Pathogenesis is presumed to be haploinsufficiency. Rio Frio et al. revealed that PRPF31 amount was significantly decreased, and truncated protein was not detected in the cells originated from the patients. Subnuclear location of unaffected protein was normal [47]. Patients having *PRPF31* mutation present highly variable penetrance [48]. Protective loci have been proposed to be associated with this incomplete penetrance and genetic modifier controls PRPF31 as CNOT3, which was suggested as disease modifier [48–50]. However, it is not consistently found and further studies are needed [51].

PRPF31 accounts for 1–10% of ADRP [36, 52–55]. It is reported as 3% in Japanese and 6–10% in Chinese ADRP patients. In Korean NGS screening, PRPF31 was most frequently found as 30% in ADRP patients whose causal variants were detected [33]. Two families had c.421-1G>A and p.R354X, respectively, and these are truncating mutations that cause null allele. These families showed variable expressivity of disease. Missense mutation is seldom reported and we had one sporadic case with p. E104K. And we had one family with missense *PRPF3* mutation (p.T494 M) [56]. The mutation was previously reported in other study and showed rapid progression like our case [57]. PRPF3 is found in 5% of ADRP patients.

9.5.3 *EYS* Gene

EYS is located in 6q12 region and inherited as AR pattern. It is relatively recently found RP gene. *EYS* is the largest gene in the eye and encodes SPAM protein that consists of 3156 amino acids in the longest isoform. SPAM have more than 21 EGF-like domains in N-terminal and 5 laminin A G-like domain in C-terminal. It is an ortholog of drosophila SPAM and named after this. Its function in human retina is not yet known, but the presence of EYS determines closure of rhabdom system and that EYS is important in integrity of rhabdom system [58]. SPAM

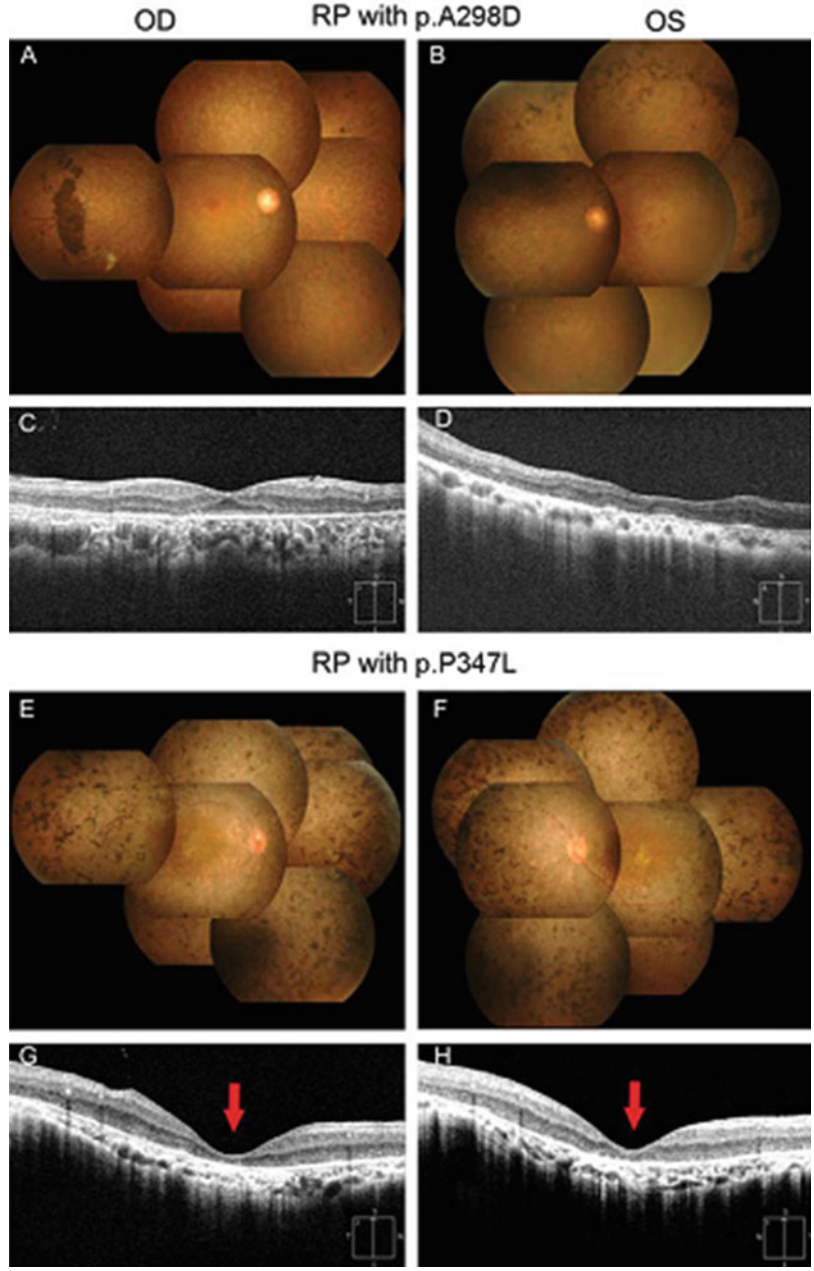

Fig. 9.1 Comparison of fundus photographs and spectral domain optical coherence tomography (SD-OCT) between patients with the p.A298D and the p.P347L mutations. Fundus photographs (**a**, **b**, **e**, **f**) show typical retinitis pigmentosa (RP) features: retinal degeneration with pigmentation, atrophy of retinal pigment epithelium (RPE), and attenuated vessels, which involved the area inside the vascular arcade. Compared with a 55-year-old patient with the p.A298D mutation (**a**, **b**), a 44-year-old patient with the p.P347L mutation (**e**, **f**) had more severe retinal pigmentation, despite being 11 years younger. SD-OCT (**c**, **d**, **g**, **h**) revealed the degeneration of photoreceptor and RPE layers and the disruption of the inner and outer segment junction of the photoreceptor in both patients. In particular, severe foveal atrophy in the patient with the p.P347L mutation resulted in a large decrease of the central foveal thickness: 161 μm in the *right* eye (**g**; *arrow*) and 152 μm in the *left* eye (**h**; *arrow*) (Reprinted from Molecular Vision 2011; 17:844–853 (Kim et al.) [14])

is mostly expressed in the retina, especially in photoreceptor of human [73].

EYS mutation accounts for 5–28% of ARRP [59–65]. Proportion has reported to be 5% in the Netherlands and Canada, 7% in Israel, 11% in the UK, 12% in France, and 17% in Spain. Interestingly it was highly reported in Japan as 23%–28%. In Korea it is the most abundant causal gene in ARRP [33]. And the vast majority of mutation was truncating. These mutations were mostly located at dispersed domain structure, and relatively many functional domains may have disease-protecting effect on missense variation. In Korean cases we have two sporadic cases.

Table 9.3 Clinical spectrum of patients having Rho mutation

	Mutation	Patient (family)	Gender	Age	Vod	Vos	OCT	VF
Cytoplasmic domain								
C-terminal tail	p.P347L	RP070(F09)	F	47	HM	HM	PR −/−	
C-terminal tail	p.P347L	RP073(F09)	M	16	1	1	PR 2.6 CME	Paracentral ring-type scotoma
C-terminal tail	p.P347L	RP071(F09)	F	20	1	0.5	PR 2.7 CME	10~20
C-terminal tail	p.P347L	RP072(F09)	F	18	1	1	PR 3, CME	Near normal
C-terminal tail	p.P347L	RP073(F09)	M	13	0.9	1	PR 4.1, CME	
C-terminal tail	p.P347L	R0358	F	35	0.6	0.7		
Transmembrane domain								
Transmembrane III	p.R135W	R0119	M	30	0.3	0.7		30/30
Transmembrane III	p.R135W	R0352	F	63	HM	FC	PR −/−	
Transmembrane VII	p.A298D	R0148	M	63	LP	LP	PR 1.3/−	
Intradiscal domain								
N-terminus (intradiscal)	p.T17M	R0363	M	54	0.6	0.7	PR 1.43/0.7	10/10
Loop IV–V (intradiscal)	p.D190N	R0513	M	68	0.5	0.5	PR 0.5/−, ERM	10/10
Loop IV–V (intradiscal)	p.D190N	591	F	52	0.1	0.1		
Loop IV–V (intradiscal)	p.Y178C	RF110(F13)	M	52	HM	NLP	PR −/−	
Loop IV–V (intradiscal)	p.Y178C	RF113(F13)	F	58	0.15	0.02	PR −, ERM	
Loop IV–V (intradiscal)	p.Y178C	RF111(F13)	M	50	0.15	0.4	PR −, CME	
Loop IV–V (intradiscal)	p.Y178C	RF112(F13)	F	25	0.8	0.6	PR 1	Temporal constriction
Loop IV–V (intradiscal)	p.Y178C	RF114(F13)	M	23	0.4	0.6	PR 2.3, cyst	

Vod vision of right eye
Vos vision of left eye
OCT optical coherence tomography
PR preserved photoreceptor inner and outer segment junction in the horizontal macular scan (OD/OS)
VF Goldmann visual field test
The visual field test result was nearly identical

They have compound heterozygote mutations, c. G1750 T/c.4958_4959insA and c. G6557A/c.4958_4959insA. Three of them were truncating mutations. One mutation (c.4958_4959insA; p.S1653 fs) was recurrently detected in two unrelated patients in our study. This mutation is in the middle of the protein near the important putative coiled-coil domain. Interestingly, c.4957_4958insA (p.S1653Kfs*2), most frequent *EYS* mutation in Japan, is also detected in two unrelated Korean patients [66]. And second common *EYS* mutation in Japan, c.8868C>A was also found in Korean patients. This was reported in Japanese study which partly recruited Korean patients. And one missense mutation (c. G6557A) was found in Korean-American [63]. This recurrent detection of same mutation might suggest that some of these mutations can be found in Koreans. Although early reports of EYS mutation showed mild

clinical features, consistent genotype-phenotype correlation has not been reported. Copy number variation (CNV) analysis using multiple ligation-dependent probe amplification revealed midsize gene rearrangement in 4% of patients in which no causal mutation was detected and 15% of patients in which the second pathogenic EYS allele was not found [67]. Therefore, thorough CNV analysis can detect more causal *EYS* mutation and proportion of *EYS* mutation in RP can be increased. Many studies noted that macular region is preserved until relatively late life. Histologic study also revealed that macula cone was relatively intact in older patients over 80s even when the rod cell was totally absent and the retina was significantly thinned. Patients with two truncating alleles are reported to progress more rapidly than the patients with one truncating mutation [59], but our cases did not show this tendency.

9.5.4 *RP1* Gene

RP1 protein is microtubule-associated protein and located at axoneme of the rod and cone outer segment along with RP1L1 [68, 69]. It is important in outer segment orientation and organization, and RP1 disruption makes progressive RP degeneration in mice. Although majority of *RP1* mutation is inherited as ADRP, AR inheritance was also reported [70]. Chen et al. classified RP mutation by four categories according to genetic location of variant [71]. Mutation in Class I is located in a.a. 1–236 and it is rare and inherited as AR pattern. Insensitive to nonsense-mediated decay (NMD), pathomechanism is not associated with haploinsufficiency. Class II comprised of mutation between a.a. 500 and 1053 and located in exon 4. This kind of mutation is NMD insensitive and dominant negative effect is proposed. Most of our cases (p.G706R, p.Q766X and p.D984G) are located in this region and showed AD inheritance pattern. Class III is located in a.a. 264–499 and 1054–1751. This class usually shows AR inheritance pattern. However, one case of our study having p.Y485X showed AR inheritance. Lastly, Class IV is mutation after a.a. 1861 and it is not pathogenic even in homozygote. Mutational hotspot is c.1490–3216 in exon 4 especially downstream from doublecortin domain, and most of Korean cases (p.G706R, p.Q766X, and p.D984G) belong to this region [71]. Two of Korean cases are premature stop codon mutation.

RP1 mutation accounts for 5.5% of ADRP and 1% of ARRP [11]. It comprises 3% of ADRP in Chinese [31]. Usually RP1 mutation brings classical phenotype of RP with relatively spared central vision. In Korean cases, one family having p.Q766X has relatively slow progression. All patients experienced their first symptoms in their 30s, and three patients who showed the first symptoms in 40s preserved normal vision.

9.5.5 X-Linked RP (*RP2* and *RPGR*)

X-linked RP (XLRP) is estimated to account for 6–20% of all RP [72]. Two principal genes, RP2 and RPGR, have been identified as the primary causative genes in XLRP. XLRP is widely reported to result in the severe forms of RP, which typically show a rapid course of vision loss, with a significant proportion progressing to legal blindness by 40s.

RP2 mutations in *RP2* are reported to cause 7–10% of XLRP [26]. RP2 is ubiquitously expressed and consists of 5 exons and 350 amino acid [73]. In human eye, RP2 is observed in plasma membrane of photoreceptors including inner and outer segment [74]. Tubulin-coating cofactor c-like (TBCC) domain in N-terminal and nucleotide diphosphate kinase in C-terminal are preserved important domains of RP2. Among associating proteins are polycystine 2, importin β2, beta subunit of rod transducin, and ADP-ribosylation factor 3 (Arl3), and Arl3 has been widely studied [75]. Exon2 including TBCC domain and Arl3 binding site is mutational hotspot where more than half of reported mutations were located [76]. Two RP2 causative variants (p.C114R and p.Ser187fs) in Koreans are also located in Arl3 binding site.

Severe clinical features and association with myopia are described in the previous section of this script. Because early macula involvement is

frequently detected, it is sometimes confused with cone-rod dystrophy [26]. The affected males experienced early onset of symptoms and presented with rapid progression in Korean cases [33]. Early macula involvement was not observed in our cases. Female carrier shows abnormalities in first examination as much as 40% [77]. Cone amplitude in ERG was decreased in two female carriers of one Korean family having *RP2* mutation while vision was normal and fundus was not consistent with typical RP.

The *RPGR* gene is known to be the major causative gene in XLRP more than 70% [72]. However, it was not detected in targeted exome sequencing study of RP in Korean. Roughly two thirds of disease-causing mutations are found in ORF15 region [72]. Phylogenetic analyses of *RPGR* revealed that ORF15 region shows rapid rate of evolution, implying that this can be mutational hotspot [78]. This repetitive purine-rich ORF15 region is rarely covered by next-generation sequencing. This technical pitfall can explain the absence of *RPGR* mutation in Korean cases. Or *RPGR* mutation might be really rare in Korean patients. Further studies are required to answer this question.

9.5.6 Genome of Korean

Korean genome analysis revealed that Korean males studied were more similar to Han Chinese than Caucasian [79]. 99.5% of short indels were identical to Han Chinese. However, 60% of single nucleotide polymorphism was different from Han Chinese. Genetic lineage map was close to Chinese and Japanese. This study supports that the genetic feature of Korean RP is similar to Chinese and Japanese rather than European.

9.6 Conclusion

Prevalence of RP in Korean is relatively similar to other East Asian ethnicities including Chinese and Japanese, rather than Caucasian-based ethnicity. Core of RP is Mendelian disorder consisting of enormously diverse genetic heterogeneity. Therefore, efforts should be focused on elucidating unique genetic feature of Korean RP patients. Next-generation sequencing is expected to be used widely in molecular diagnosis of RP. If traits of genetic variability in Korean RP patients are well established, narrowing down the target and decreasing the cost burden could be possible in next-generation sequencing. Gene replacement therapy, which is studied vigorously recently, also depends on the detailed genetic profile of a patient to become feasible.

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Retinitis Pigmentosa in China

10

Ruifang Sui and Xuan Zou

Abstract

Retinitis pigmentosa is clinically and genetically heterogeneous, with about 80 genes implicated so far. The patients typically lose night vision in adolescence, side vision in young adulthood, and central vision in later life because of progressive loss of rod and cone photoreceptor cells. China starts genetic screening for RP in 1990s, with very different causative genes and mutation spectrum compared to that of the Western population. In recent years, several novel RP genes were identified in Chinese patients. The results expand the genetic spectrum of non-syndromic retinal degeneration. Imminent treatments for retinitis pigmentosa are greatly anticipated, especially for genetically defined subsets of patients, because of newly identified genes, growing knowledge of affected biochemical pathways, and development of animal models.

Keywords

Chinese • Retinitis pigmentosa • Next-generation sequencing • Phenotype • Genotype

10.1 Introduction

Inherited retinal degeneration (RD) is a leading cause of blindness in many parts of the world. RD affects approximately 1 in 2000 people worldwide. Retinitis pigmentosa (RP) is the most common and highly heterogeneous form of RD. RP may occur alone, as non-syndromic RP without other clinical findings or as syndromic or systemic RP with other neurosensory disorders, developmental abnormalities, or complex clinical phenotypes. This section is limited to non-syndromic RP. Patients with RP usually start from loss of peripheral vision and night vision and, gradually, the central portion of vision is affected and finally loss of all fields of vision. RP can be inherited in different patterns, and the autosomal recessive is the most common pattern, followed by autosomal dominant and X-linked

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inheritance. RP has many disease-causing genes, many known mutations, and highly varied clinical consequences. Identification and understanding the disease-causing gene mutations is the key to develop new treatment and provide counseling for the patients and their families.

10.2 Epidemiology

RP has a worldwide incidence of approximately one in 3500–5000 individuals. Approximately 10–19% of the cases are autosomal dominant (adRP), 6–8% are X-linked (xlRP), and the remaining cases are most likely autosomal recessive (arRP) [1–3]. Non-Mendelian inheritance patterns, such as digenic inheritance and maternal (mitochondrial) inheritance, have been reported but probably account for only a small proportion of cases.

10.3 China Perspective

The reported incidence of RP in China is around 1/3800 [4], resulting in an estimated total of 0.4 million Chinese RP patients. The proportion of RP from Peking Union Medical College Hospital is as Fig. 10.1, which is similar to the reported results in western population.

It is known that both causative genes and mutation spectrums vary dramatically among different ethnic groups. For example, mutations in *RHO* are much less frequent in Chinese RP patients than in RP patients of European descent; similarly, the mutation spectrums of several genes such as *RPE65*, *USH2A*, and *EYS* among Asian patients are different from those among European Caucasians.

China starts genetic screening for RP in 1990s. With the emergence of next-generation sequencing (NGS), comprehensive molecular diagnosis of RP is becoming a reality. In a recent study involving 60 RP-related genes screening in 157 unrelated Chinese families with RP, mutations in 50% of the 157 families were confirmed. The genes that most frequently harbored mutations compared to the other genes that are associated with autosomal dominant, autosomal recessive, and X-linked RP were *RHO*, *USH2A*, and *RPGR* genes, respectively (Fig. 10.2) [5].

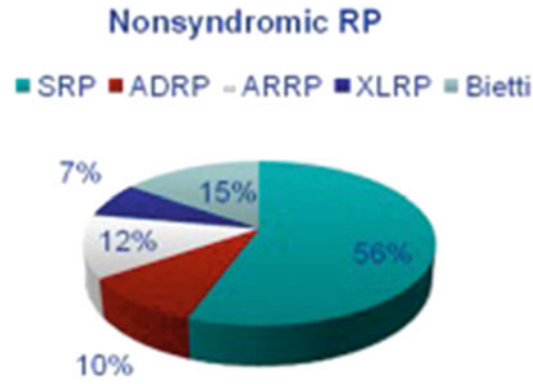

Fig. 10.1 The proportion of non-syndromic RP of patients from Peking Union Medical College Hospital

Recently, several novel RP genes were identified in Chinese patients. *IFT140* variants were identified in multiple unrelated non-syndromic Leber congenital amaurosis (LCA) and RP cases. The results expand the phenotype spectrum of *IFT140* mutations to non-syndromic retinal degeneration, thus extending our understanding of intraflagellar transport and primary cilia biology in the retina [6]. *SPP2* and *HK1* were found to be very likely novel adRP-causing genes [7, 8]. *HK1* is the first report that associates the glucose metabolic pathway with human retinal degenerative disease, suggesting a potential new disease mechanism. Finally, a potential novel genotype-phenotype correlation was found between *NEUROD1* and non-syndromic arRP [9].

10.4 Etiology and Pathology

RP-related genes function in strikingly diverse biological pathways, including phototransduction, the retinoid (vitamin A) cycle, gene transcription, RNA splicing, photoreceptor structure, and so on. At cellular level, a simplified view of RP is progressive dysfunction and loss of rod photoreceptors, first affecting night vision in the rod-rich mid-peripheral retina, then progressing into the cone-rich central retina, with eventual loss of cones either as a direct

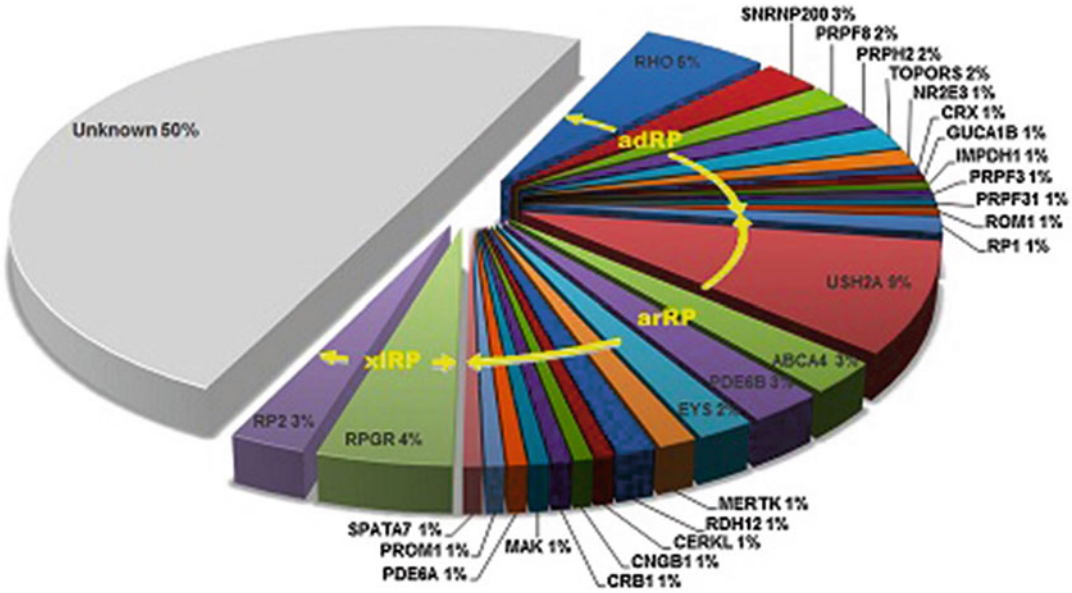

Fig. 10.2 Mutation spectrum of individual genes in the 157 unrelated patients with RP

result of the disease process or secondary to the death of rods.

10.5 Clinical Features

RP is a group of hereditary retinal degeneration diseases that are characterized by night blindness, a constriction of the visual field, a gradual reduction of the visual acuity, waxy pale optic discs, attenuated retinal artery, and pigment abnormality initially from the mid-peripheral retina. The most common pigment abnormality is black bone-spicule pigmentation due to the death of retinal pigment epithelium (RPE). Clinically, patients usually start from loss of peripheral vision and night vision and, gradually, the central portion of vision is affected and finally loss of all fields of vision. Electroretinogram (ERG) recordings are usually extinguished or severely reduced, with the rods being affected before the cones. By midlife, RP patients may retain a few degrees of central vision, but in many cases the disease culminates in complete blindness. Within this broad picture though, there is considerable variation in age of onset, rate of progression, rod vs cone involvement, involvement of other retinal cells such as RPE, secondary symptoms such as cystic macular edema, and many other features. The diversity of the clinical appearances of RP is partially due to the underlying genetic heterogeneity of the disease.

10.6 Genetic Aspects

Molecular genetic studies of RP have produced great progress in recent years. To date, at least 77 genes have been reported to be associated with non-syndromic RP as listed in RetNet (http://www.sph.uth.tmc.edu/retnet/). Mutations in 27 genes are known to cause autosomal-dominant RP, 55 genes cause recessive RP, and 3 genes cause X-linked RP [10]. These genes account for only about 60% of all patients; the remainder have defects in as yet unidentified genes. If genes for LCA and for other syndromic or systemic forms of RP are included, at least 100 "RP-related" genes are known. Allelic or mutational heterogeneity is equally striking. Counting all the genes known to cause non-syndromic RP, nearly 3100 disease-causing mutations are reported in mutation databases [10]. Of these genes, mutations in *RHO*, *USH2A*, and *RPGR* were the most frequently reported in previous studies to be responsible

for approximately 30% of all of the RP cases [11]. However, because of the highly heterogeneous nature of RP and the potential ethnic variety of individual gene variations, genes that are frequently mutated in western population, such as *RHO* and *CYP4V2,* are not common causes in China [12, 13].

10.7 Laboratory

Progress in finding treatments is dependent on determining the genes and mutations causing these diseases, which includes both gene discovery and mutation screening in affected individuals and families. The standard techniques for gene discovery and mutation detection – linkage mapping and DNA sequencing – have been used for many years. However, development of high-density and high-throughput techniques in the past 10 years has increased the power of these methods by orders of magnitude.

One of the most powerful approaches to genetic testing is high-throughput "deep sequencing," that is, NGS. NGS does millions of sequencing runs in parallel on micron-sized beads or in comparable micro-wells, completing up to a billion base-pair reads per run. That is, NGS sequencing is at least 1000 times faster than conventional sequencing and much less expensive per sequence. Broadly, there are three NGS strategies: whole-exome NGS, whole-genome NGS, and targeted-capture NGS. NGS has identified several novel RP genes.

Finally, some mutations are not easily detected by conventional sequencing or NGS, particularly large deletions and rearrangements. Some deletions can be detected by SNP arrays, and the Affymetrix 6.0 SNP/CNV arrays include copy-number probes (CNVs) for deletion detection. PCR amplification-based methods, such as MLPA or qPCR, can detect much smaller deletions.

10.8 Summary

Gene therapy for retinal disorders is poised to become a clinical reality. Accurate molecular diagnosis of RP is critical for the diagnosis, prognosis, management, and eventually, treatment of patients. The majority of genetic studies on RD were conducted in patients of European descent, and only a small subset of known RP disease genes have been investigated in Chinese patients. Nevertheless, gene spectrums vary dramatically among different ethnic groups. Therefore, different study groups in China now are working on the genetic screening RP, associated biochemical defects, and even the gene therapy in animal models. The research findings will lay the foundation for developing new diagnosis approach and treatment methods. The studies in Chinese population will benefit all RP patients worldwide.

Compliance with Ethical Requirements Ruifang Sui and Xuan Zou declare no conflict of interest.

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

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Retinitis Pigmentosa in Japanese Population

11

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Abstract

Retinitis pigmentosa (RP) is a highly heterogeneous genetic disease with autosomal recessive (ar), autosomal dominant, or X-linked inheritance. It is known that *EYS* (eyes shut homolog) and *USH2A* genes account for 5–16% and 7–23% arRP patients, respectively, which was reported using mainly Caucasian patients. We analyzed mutations of *EYS* and *USH2A* in Japanese ar and isolated non-syndromic RP patients (total 100) as the first analysis. All exons of these genes were analyzed by Sanger sequencing. The analysis of the *EYS* in 100 RP patients elucidated 7 very likely pathogenic mutations from 18 patients. Of them, 12 had a particular insertion mutation c.4957_4958insA and 4 had a nonsense mutation c.8868C>A. These 2 mutations were considered frequent mutations in Japanese RP patients. The minimum observed prevalence of *EYS* mutations was 18%, which is higher than previously reported with Caucasians. The RP patients in whom *EYS* mutations were not detected (82 patients) were screened for *USH2A*. We found 5 very likely pathogenic mutations in 4 patients (4%), which was lower than reported previously. Most mutations found in these 2 genes in Japanese RP patients were novel, and specific frequent mutations for each gene were found, strongly suggesting that the mutational spectra of these genes differ markedly from Caucasians. Based on these data, if both the *EYS* and *USH2A* genes are analyzed among Japanese arRP patients, gene defects could be detected in approximately 22% of patients (18% and 4%, respectively). We suggest that screening for these 2 genes will be effective in genetic testing and for counseling Japanese patients with RP.

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Keywords
Retinitis pigmentosa • Inherited retinal disease • Mutation analysis • *EYS* • *USH2A* • Mutation spectrum • Japanese population

11.1 Introduction

Retinitis pigmentosa (RP, Mendelian Inheritance in Man [MIM 268000]) is the most common form of hereditary retinal degeneration, and its prevalence rate is thought to be approximately 1 in 5000 people worldwide [11] and 1 in 4000–8000 people in Japan. RP is a genetically and clinically heterogeneous disease. RP involves progressive retinal degeneration characterized by night blindness and visual field constriction, which eventually leads to severe visual impairment.

RP can be divided into two groups: non-syndromic RP in which RP is restricted to the eyes without other systemic manifestations and syndromic RP in which patients present with other associated neurosensory disorders or complex clinical phenotypes. Approximately 20–30% of patients with RP are classified as having syndromic RP [14]. For example, Usher syndrome (USH) is the most common form of syndromic RP and presents with congenital or early onset deafness.

RP can be inherited as an autosomal dominant (ad: 30–40%), autosomal recessive (ar: 50–60%), or X-linked recessive (5–15%) mode in Caucasian patients [21]. The relative frequencies of RP inheritance patterns in Japanese patients have been estimated as 25.2% for ar, 16.9% for ad, 1.6% for X-linked, and 56.3% for simplex (isolated) RP; thus, most Japanese RP patients have arRP or isolated cases [22]. To date, 78 causative genes for RP have been identified (https://sph.uth.edu/retnet/; accessed August 25, 2015). Mutations in these genes account for approximately 36–60% of all RP cases, with the frequency of mutation carriers varying between different populations and ethnicities [15, 21, 45, 49].

In this chapter, we summarize the spectrum and frequency of gene mutations in Japanese patients with RP. Recently, we and other groups showed that mutations in the *EYS* and *USH2A* genes are the most frequent implicated genes in RP, accounting for approximately 13–33% and 4–7% of Japanese arRP patients, respectively [4, 23, 25, 30, 49, 67]. For the purpose of this study, we focused on the mutation analysis for various causative genes including *EYS* and *USH2A* using multiple methods.

11.2 Patients and Clinical Evaluation

Previously, we screened all *EYS* and *USH2A* exons in unrelated Japanese RP patients (total 100) with no systemic manifestations, excluding families with obvious autosomal dominant inheritance [23, 58, 67]. Some pedigrees showed a pattern compatible with the recessive mode of inheritance; other patients were to be considered isolated cases. In addition, 200 unrelated and non-RP Japanese individuals were screened as controls. Clinical diagnosis of RP was based on the visual field, fundus examinations, and electroretinogram findings. An audiological examination, including pure-tone audiometry, was not performed before the mutation analysis; however, none of the patients had documented hearing loss [23, 58, 67].

11.3 Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Hamamatsu University School of Medicine, Japan; RIKEN Center for Developmental Biology, Japan; Institute of Biomedical Research and Innovation Hospital, Japan; Nagoya University Graduate School

of Medicine, Japan; National Center for Child Health and Development, Japan; Chiba University Graduate School of Medicine, Japan; and Kyungpook National University Hospital, Korea) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

11.4 *EYS* Mutation Analysis of Japanese Patients with arRP

The *EYS* gene encodes an ortholog of *Drosophila* spacemaker and a protein essential for maintaining photoreceptor morphology. *EYS* spans over 2 Mb in the genome, making it one of the largest genes known to be expressed in the human eye [1, 12]. *EYS* mutations have been detected in arRP-affected families of different ancestral origins and are reported to account for 5–16% of arRP cases [2, 5, 7, 36]. We performed Sanger sequencing for all *EYS* exons in 100 unrelated Japanese arRP patients.

11.4.1 Mutation Analysis

Mutation analysis of *EYS* in 100 unrelated Japanese patients revealed 7 very likely pathogenic mutations in 18 patients (18%). Of these 18 patients, second mutant alleles could not be detected in 9 patients. The very likely pathogenic mutations consisted of 3 truncating mutations, 1 deletion mutation, and 3 missense mutations (Table 11.1) [2, 4, 23, 25, 29, 30, 36, 49].

A truncating insertion, c.4957_4958insA, was detected in 12 patients and accounted for 15 of the 35 mutated alleles detected (42.9%), as shown in Table 11.1 [23]. Three patients were homozygous for the c.4957_4958insA mutation, and the other 9 patients were heterozygous. Of the latter, 3 patients showed a second mutation, while 6 did not. This insertion creates a frameshift mutation that is predicted to cause a premature stop at codon 1654 (p. S1653KfsX). A truncating nonsense mutation c.8868C>A (p. Y2956X) was also identified in 4 patients and accounted for 5 of the 35 mutated alleles detected (14.3%). Thus, these 2 truncating mutations were identified in 16 separate patients, representing a very high frequency of the 2 mutations in Japanese arRP patients.

Nine of the 18 patients bearing very likely pathogenic mutations appeared to have both alleles affected, suggesting that they received one mutated allele from each unaffected parent (Table 11.1).

The remainder of the patients comprising the group with very likely pathogenic mutations presented only single truncating mutations (Table 11.1). The patients are described more in Sect. 11.7 (See Fig. 11.1). None of these seven very likely pathogenic mutations were found in the Japanese control subjects.

11.4.1.1 Screening for the Two Truncating Mutations

We focused on 2 truncating mutations, c.4957_4958insA and c.8868C>A, which were identified in 16 separate Japanese arRP patients in our study. The frequency of the 2 mutations was very high in this Japanese arRP cohort. However, we did not detect these mutations in 19 Japanese adRP patients or 28 Leber congenital amaurosis (LCA) patients who were recruited and screened to evaluate the frequency of the mutations. We also recruited 32 unrelated Korean arRP patients and screened for the 2 *EYS* mutations. The c.4957_4958insA mutation was detected in 2 patients and accounted for 3 of 64 Korean patient alleles (4.7%). One patient was homozygous and the other was heterozygous. The c.8868C>A mutation was identified in 1 patient and accounted for 1 of the 64 Korean patient alleles (1.6%).

11.4.1.2 Summary of Findings Regarding *EYS* Mutations

Mutation analysis of the *EYS* gene in 100 Japanese patients revealed 7 very likely pathogenic mutations in 18 patients (18%). The estimated prevalence in our study is higher than that found in previous studies [2, 5, 7, 36]. Although further analysis of all *EYS* exons is required, *EYS* mutations may not be detected in Japanese

Table 11.1 Mutation spectrum of the *EYS* gene among Japanese families

Family ID	Nucleotide change	Predicted effect	Exon number	State	References
Families with very likely pathogenic mutations and both alleles affected					
RP3H[a]	c.4957_4958insA	p.S1653KfsX	Exon 26	Homozygous	[4, 23, 25, 29, 30, 49]
RP48K[a]	c.4957_4958insA	p.S1653KfsX	Exon 26	Homozygous	[4, 23, 25, 29, 30, 49]
RP54K	c.4957_4958insA	p.S1653KfsX	Exon 26	Homozygous	[4, 23, 25, 29, 30, 49]
RP44K	c.4957_4958insA	p.S1653KfsX	Exon 26	Heterozygous	[4, 23, 25, 29, 30, 49]
	c.6557G>A	p.G2186E	Exon 32	Heterozygous	[2, 4, 23, 36, 49]
RP56K[a]	c.4957_4958insA	p.S1653KfsX	Exon 26	Heterozygous	[4, 23, 25, 29, 30, 49]
	c.8351T>G	p.L2784R	Exon 44	Heterozygous	[4, 23]
RP87N	c.4957_4958insA	p.S1653KfsX	Exon 26	Heterozygous	[4, 23, 25, 29, 30, 49]
	c.7793G>A	p.G2598D	Exon 40	Heterozygous	[23, 49]
RP81K[a]	c.2522_2523insA	p.Y841X	Exon 16	Heterozygous	[4, 23]
	c.6557G>A	p.G2186E	Exon 32	Heterozygous	[2, 4, 23, 36, 49]
RP21H	c.6425-?_6571+?del	p.D2142_S2191delinsG	Exon 32	Homozygous	[23]
RP35K	c.8868C>A	p.Y2956X	Exon 44	Homozygous	[4, 23, 25, 29, 49]
[b]Families with single very likely pathogenic mutations					
RP1H	c.4957_4958insA	p.S1653KfsX	Exon 26	Heterozygous	[4, 23, 25, 29, 30, 49]
RP6H	c.4957_4958insA	p.S1653KfsX	Exon 26	Heterozygous	[4, 23, 25, 29, 30, 49]
RP12H	c.4957_4958insA	p.S1653KfsX	Exon 26	Heterozygous	[4, 23, 25, 29, 30, 49]
RP51K	c.4957_4958insA	p.S1653KfsX	Exon 26	Heterozygous	[4, 23, 25, 29, 30, 49]
RP96H	c.4957_4958insA	p.S1653KfsX	Exon 26	Heterozygous	[4, 23, 25, 29, 30, 49]
RP100N	c.4957_4958insA	p.S1653KfsX	Exon 26	Heterozygous	[4, 23, 25, 29, 30, 49]
RP8H	c.8868C>A	p.Y2956X	Exon 44	Heterozygous	[4, 23, 25, 29, 49]
RP25H	c.8868C>A	p.Y2956X	Exon 44	Heterozygous	[4, 23, 25, 29, 49]
RP80K[a]	c.8868C>A	p.Y2956X	Exon 44	Heterozygous	[4, 23, 25, 29, 49]

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence NM_001292009

The initiation codon was designated as codon 1. None of these seven mutations were found in Japanese control subjects

[a]Segregation analysis was performed for the indicated patients. For the remaining patients, segregation analysis could not be performed due to difficulties in collecting samples from the families of patients

[b]The indicated patients are described more in Sect. 11.7

A separate research group demonstrated that compound heterozygous mutations consisting of c.4957_4958insA and c.8868C>A can cause of ar cone-rod dystrophy [29]

adRP or LCA patients. The c.4957_4958insA and c.8868C>A mutations were also detected in Korean arRP patients. Similar to Japanese arRP results, the c.4957_4958insA mutation was more frequently detected than the c.8868C>A mutation. The fact that both mutations were also detected in Korean patients suggests the possibility that the mutations occurred in an ancient common ancestor and spread throughout East Asia. These results indicate that the *EYS* mutation spectrum among Japanese patients largely differs from that among the previously mentioned Caucasian populations. For additional details regarding the experimental procedures and clinical findings described in this paragraph, see reference [23, 58].

11.5 *USH2A* Mutation Analysis in Japanese Patients with arRP

A separate study was conducted to determine the spectrum and frequency of *USH2A* mutations in Japanese arRP patients. The *USH2A* gene, which accounts for approximately 74–90% of USH type 2 (USH2) cases, is also one of the major causative genes associated with arRP among Caucasian populations [20, 21, 39, 40]. To identify

disease-causing *USH2A* mutations in Japanese RP patients, all 73 exons in this gene were screened for mutations by Sanger sequencing. Of the 100 RP patients described in the previous paragraph, 82 were included in this study after excluding 18 RP patients with *EYS* mutations. Here, we present the results of our study of all *USH2A* exons in Japanese arRP patients.

11.5.1 Mutation Analysis

Our mutation analysis of the *USH2A* in 82 unrelated Japanese patients revealed 5 very likely pathogenic mutations among 4 patients. One patient had only 1 very likely pathogenic mutation and the others had 2 of them (Table 11.2). These very likely pathogenic mutations consisted of a deletion mutation, 2 splicing mutations, and 2 missense mutations [35, 39, 40, 49, 67]. None of these 5 very likely pathogenic mutations were found among the Japanese control subjects. The p. C759F mutation implicated in arRP [51] and the p. E767Sfs mutation implicated in USH2 [64], which are frequently found in Caucasian populations, were not found in any of the Japanese patients studied. In our recent analysis of the *USH2A* gene in Japanese USH2 patients, we reported 19 novel mutations among 19 patients, including the splicing mutation c.8559-2A>G in 4 of 19 patients [39, 40], suggesting a possible frequent *USH2A* mutation among the Japanese population. Here, we found that patient RP15H was homozygous for the c.8559-2A>G mutation, supporting the possibility of a frequent *USH2A* mutation among the Japanese population.

11.5.1.1 Summary of Findings Regarding *USH2A* Mutations

Mutation analysis of the *USH2A* gene revealed 5 very likely pathogenic mutations in 4 patients. The prevalence of *USH2A* mutations was 4% and the profile of the *USH2A* mutations differed largely between Japanese patients and previously reported Caucasian populations. For additional details regarding the experimental procedures and clinical findings discussed in this paragraph, see reference [67].

Table 11.2 Mutation spectrum of the *USH2A* gene among Japanese families

Family ID	Nucleotide change	Predicted effect	Exon number	State	References
Families with very likely pathogenic mutations and both alleles affected					
RP7H[a]	c.685G>C	p.G229R	Exon 4	Heterozygous	[67]
	c.3595_3597delGAA	p.E1199del	Exon 17	Heterozygous	[67]
RP10H	c.685G>C	p.G229R	Exon 4	Heterozygous	[67]
	c.2776C>T	p.R926C	Exon 13	Heterozygous	[67]
RP15H	c.8559-2A>G[b]		Intron 42	Homozygous	[39, 40, 49, 67]
Families with single very likely pathogenic mutations					
RP66K	c.486-14G>A[c]		Intron 2	Heterozygous	[35, 67]

Nucleotide numbering reflects cDNA numbering with +1 corresponding to A of the ATG translation initiation codon in the reference sequence NM_206933

The initiation codon was designated as codon 1. None of the indicated five mutations were found in Japanese control subjects

[a]Segregation analysis was performed for the indicated patients. For the remaining patients, segregation analysis could not be performed due to difficulties in collecting samples from the families of patients

[b]We demonstrated that compound heterozygous mutations involving c.8559-2A>G or homozygous c.8559-2A>G mutations can cause Usher syndrome type 2 (USH2) [39, 40, 67]

Usher syndrome (USH) can be clinically classified into three subtypes on the basis of severity and progression of hearing loss and the presence or absence of vestibular dysfunction

Specifically, USH2, the most common type accounting for >50% of USH cases, is characterized by congenital mild-to-severe hearing loss and a normal vestibular response

[c]c486-14G>A was previously reported as disease causing in a French USH2 patient and shown to create a new AG (acceptor consensus) sequence, resulting in abnormal splicing [35]

11.6 Summary of *EYS* and *USH2A* Mutation Analyses with Japanese arRP Patients

We screened all *EYS* exons in 100 unrelated Japanese arRP patients and detected very likely pathogenic *EYS* mutations in 18% of the patients [23]. Excluding 18 RP patients with *EYS* mutations, 82 of these 100 patients were employed in next study. Among them, we found at least 1 very likely pathogenic *USH2A* mutation in 4 cases [67]. The mutation spectrum of the *EYS* and *USH2A* genes in Japanese arRP patients largely differed from the previously reported spectrum in patients from Caucasian populations. Based on these data, if both *EYS* and *USH2A* are analyzed among Japanese arRP patients, gene defects could be detected in 22% of the patients. RP is a highly heterogeneous disease, with a reported prevalence rate of 1 in 4000–8000 people in Japan. Given the population of Japan, approximately 100 million, the number of patients with RP can be estimated to be 12,500–25,000. Autosomal recessive and simplex cases account over 80% of RP cases in Japan (approximately 10,000–20,000 people). Our results indicate that mutation analysis for the *EYS* and *USH2A* genes can potentially provide diagnosis for approximately 2200–4400 Japanese RP patients. Therefore, screening for these 2 genes may be very useful for genetic testing and counseling, and analysis of the mutated proteins may be helpful in the development of effective therapies for RP patients in Japan. Below, we discuss 2 more topics, namely, multiplex ligation-dependent probe amplification (MLPA) analysis and the mutation spectrum of causative RP genes in Japanese RP patients.

11.7 Copy Number Variation Analysis of Each Exon Within *EYS* in arRP Patients

We used MLPA to detect mutations that cannot be easily identified by Sanger sequencing. MLPA can detect not only point mutations but also copy number alterations [57]. We analyzed 9 RP patients who showed an *EYS* mutation in only 1 allele (Table 11.1). Of these, 2 patients (RP8H and RP12H) showed significant results (Fig. 11.1a-1, b-1). In the case of patient RP8H, 3 copy equivalent signal was found in exon 23, suggesting duplication of 1 allele of exon 23. In another case (patient RP12H), a single-copy equivalent signal in exon 1 was observed. This finding suggested that 1 allele of exon 1 was deleted. In addition, these mutations were found to co-segregate with disease phenotypes by segregation analysis (Fig. 11.1a-2, b-2). Consequently, copy number alterations possibly contribute as compound heterozygous mutations. It is possible that more of copy number mutations exist latently in *EYS*, *USH2A*, and other causative RP genes.

11.8 The Mutation Spectrum of Causative RP Genes in Japanese RP Patients

Since the discovery of *RHO*, the first gene implicated in RP [13], more than 70 genes were detected as the cause of RP, and numerous pathogenic mutations have been published subsequently. Since the 1990s, many research groups in Japan energetically have performed molecular and mutation analysis for Japanese RP patients [4, 9, 10, 16–19, 23–34, 37–44, 47, 49, 52–56, 60–62, 66, 67]. These pathogenic mutations are deposited in private databases and have not yet been made available to the public. As a final topic, we performed a literature search for publically reported mutations in Japanese patients with RP. Here, we provide an overview of the spectrum of RP mutations in the Japanese population (Table 11.3). For simplicity, this review is limited to non-syndromic RP. To the best of our knowledge, more than 198 different mutations have been extracted in 37 known causative RP genes. Table 11.3 shows that various types of *EYS* mutations, including 2 frequent mutations, have been detected by other studies in Japanese patients [4, 23, 25, 29, 30, 49]. Therefore, these data strongly suggest that *EYS* mutations play a

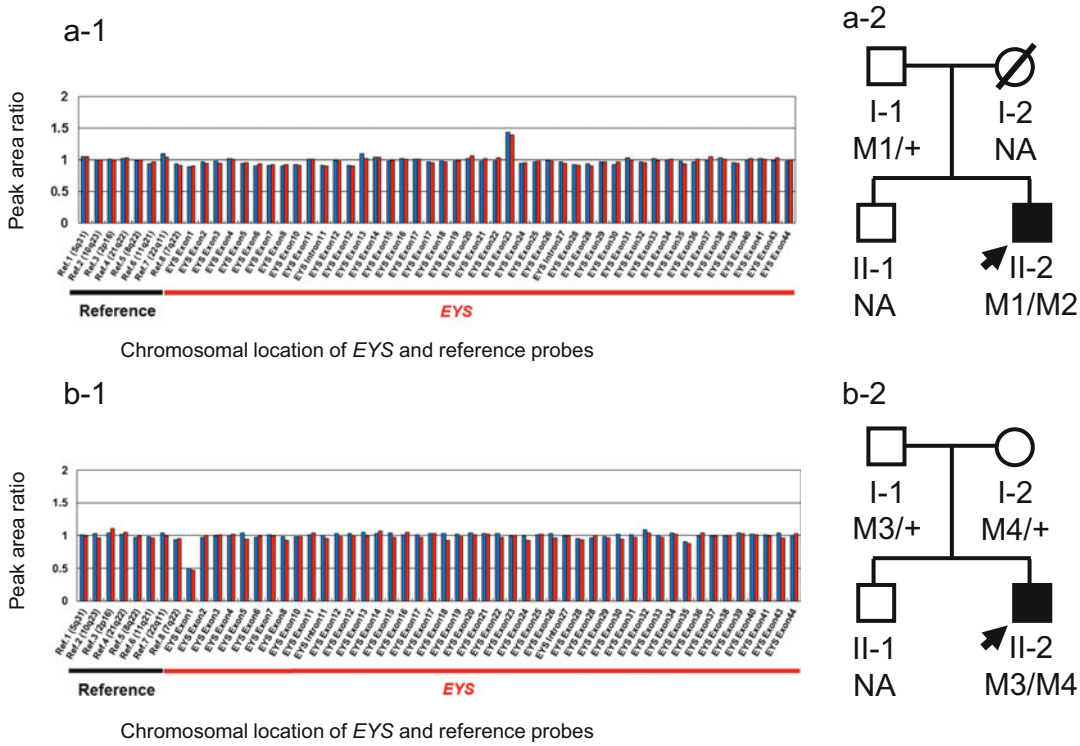

Fig. 11.1 Results of MLPA analysis and pedigree of patients with copy number mutations in the *EYS* gene. Histograms of peak area ratios for RP8H (**a**-1) and RP12H (**b**-1). The height of the columns represents the dosage of the respective segments in genomic DNA with two alleles, where a value of about one corresponds to two alleles. The X-axis shows the chromosomal locations of the *EYS* and reference (control) probes. A peak area ratio of 1.0 represents no copy number change. Peak area ratios falling outside the range 0.7–1.3 times the control peak area were considered abnormal, with those below 0.7 representing deletions and those above 1.3 representing duplications. The *EYS* reference sequence used for this study is NM_001292009. The pedigrees of patients RP8H (**a**-2) and RP12H (**b**-2) are shown. The genotypes are represented for either c.8868C>A (M1), a duplication in exon 23 (M2), c.4957_4958insA (M3), or a deletion in exon 1 (M4). The genotype of each evaluated individual is indicated below the symbol: *square boxes* and *circles* denote male and female individuals, respectively; *black* symbols indicate affected individuals; and a *slashed* symbol indicates a deceased individual. The probands are indicated with *arrows*. NA, unavailable DNA samples. For example, M1/+, heterozygous carriers; M1/M2, individuals who were compound heterozygous for both mutations. We used the commercially available SALSA MLPA P328-A1 Kit (MRC Holland), according to the manufacturer's instructions. Electrophoresis of PCR products was performed using an ABI PRISM 3110 instrument (Applied Biosystems). Data analysis was performed after exporting the values of peak sizes and areas to a Microsoft Excel file. Each result was confirmed by two independent tests, which are represented by the *blue* and *red* columns, respectively

major role in the pathogenesis of RP affecting the Japanese population.

11.9 Future Directions

With continual advances in sequencing techniques, significant progress has been made in mutation screening for patients. It is plausible that molecular diagnosis for patients will become a routine procedure in clinical practice in the coming years. However, a major question is how molecular diagnostic results for patients will be interpreted. It is challenging to predict the pathogenic effects for many genetic variations. Functional assays would be needed to characterize each variation, but this approach would be difficult to achieve in routine benchwork. Each of the genetic variations in our reports was evaluated for its pathogenicity

Table 11.3 The mutation spectrum of causative RP genes in Japanese population

Gene	Accession No.	Mode of inheritance	Nucleotide change	Predicted effect	Location in gene	References
ABCA4	NM_000350	ar	c.859-45_952delinsTCTGACC		Intron 7	[19]
			c.1760+2T>G[b]		Exon 12	[19]
[a]*BEST1*	NM_004183	ad	c.763C>T[c]	p.R255W	Exon 7	[49, 65]
C2orf71	NM_001029883	ar	c.85C>T	p.R29W	Exon 1	[30]
			c.2126delG	p.G709Efs	Exon 1	[49]
			c.2988dupC	p.T997Hfs	Exon 1	[49]
			c.3748C>T	p.R1250C	Exon 2	[30]
CNGA1	NM_000087	ar	c.191delG	p.G64VfsX29	Exon 5	[30, 49]
			c.265delC	p.L89FfsX4	Exon 6	[30]
			c.839G>A	p.R280H	Exon 11	[49]
			c.989A>G	p.D330G	Exon 11	[49]
			c.1429delG	p.V477YfsX17	Exon 11	[30]
			c.1633G>A	p.G545S	Exon 11	[49]
CNGB1	NM_001297	ar	c.217+5G>C		Intron 3	[49]
			c.2524_2525insA	p.G842Nfs	Exon 26	[49]
			c.3462+1G>A		Intron 32	[34]
CRB1	NM_201253	ar	c.2972A>T	p.H991L	Exon 9	[28]
CRX	NM_000554	ad	c.193G>C	p.D65H	Exon 3	[28, 49]
			c.455C>A	p.S152Y	Exon 4	[28]
			c.897G>C	p.L299F	Exon 4	[49]
DFNB31	NM_001083885	ar	c.160C>T	p.Q54X	Exon 6	[47]
EYS	NM_001292009	ar	c.10A>G	p.K4E	Exon 4	[4]
			c.35T>C	p.M12T	Exon 4	[4]
			c.77G>A	p.R26Q	Exon 4	[4, 23]
			c.141A>T	p.E47D	Exon 4	[4, 30]
			c.179delT	p.L60Wfs	Exon 4	[4]
			c.228G>C	p.Q76H	Exon 4	[4]
			c.632G>A	p.C211Y	Exon 4	[4]
			c.768A>G	p.I256M	Exon 5	[4]
			c.1211dupA	p.N404Kfs	Exon 8	[25]

			c.1345A>T	p.K449X	Exon 9	[49]
			c.1450G>A	p.G484R	Exon 9	[4]
			c.1485_1493delGGTTATTGAinsCGAAAAG	p.V495Efs	Exon 10	[25]
			c.1750G>T	p.E584X	Exon 11	[49]
			c.2380C>T	p. R794X	Exon 15	[4]
			c.2522_2523insA	p.Y841X	Exon 16	[4, 23]
			c.2923T>C	p.C975R	Exon 19	[23]
			c.3489T>A	p.N1163K	Exon 23	[25]
			Invert-dup Exon 23–29		Exon 23–29	[47]
			c.3614A>C	p.N1205T	Exon 24	[4]
			c.3809T>G	p.V1270G	Exon 25	[25]
			c.4022delC	p.S1341Ffs	Exon 26	[30]
			c.4387delA	p.R1463Gfs	Exon 26	[49]
			c.4402_4403insTCAAGAGG	p.D1468Vfs	Exon 26	[49]
			c.4557delA	p.A1520Pfs	Exon 26	[4]
			c.4897A>G	p.K1633E	Exon 26	[4]
			c.4957_4958insA[d]	p.S1653Kfs	Exon 26	
			c.4963T>A	p.L1655M	Exon 26	[4]
			c.5014C>T	p.Q1672X	Exon 26	[49]
			c.5168_5169insT	p.L1723Ffs	Exon 26	[47]
			c.5202_5203delGT	p.F1735Qfs	Exon 26	[4, 49]
			c.5404C>T	p.L1802F	Exon 26	[4, 23]
			c.5836-2A>G		Intron 27	[4]
			c.5836-3_4insT		Intron 27	[4]
			c.5884A>G	p.T1962A	Exon 28	[23]
			c.6425-?_6571+?del	p.D2142_S2191delinsG	Exon 32	[23]
			c.6557G>A	p.G2186E	Exon 32	
			c.6714delT	p.P2238Pfs	Exon 33	[30]
			c.6897_6902dupAGGTCC	p.G2300_P2301dup	Exon 35	[4]
			c.6976C>T	p.R2326X	Exon 35	[4]
			c.7002C>A	p.C2334X	Exon 35	[30]
			c.7028_7029delTGinsATCGT	p.L2343Hfs	Exon 35	[25]
			c.7048delT	p.C2350Afs	Exon 35	[49]
			c.7283C>A	p.S2428X	Exon 37	[25]

(*continued*)

Table 11.3 (continued)

Gene	Accession No.	Mode of inheritance	Nucleotide change	Predicted effect	Location in gene	References
			c.7579-1G>T		Intron 38	[4]
			c.7665_7666delCA	p.Y2555X	Exon 39	[25]
			c.7694delA	p.N2565Mfs	Exon 39	[49]
			c.7793G>A	p.G2598D	Exon 40	[23, 49]
			c.7810C>T	p.R2604C	Exon 40	[4]
			c.7836_7837delTC	p.P2613Lfs	Exon 40	[4]
			c.7919G>A	p.W2640X	Exon 41	[49]
			c.8012T>A	p.L2671X	Exon 41	[49]
			c.8196_8200delCTTTC	p.F2733Cfs	Exon 43	[4]
			c.8299G>C	p.D2767H	Exon 44	[4]
			c.8351T>G	P.L2784R	Exon 44	[4, 23]
			c.8439_8442dupTGCA	p.E2815Cfs	Exon 44	[4]
			c.8442_8443insTG	p.E2815Wfs	Exon 44	[49]
			c.8868C>A[d]	p.Y2956X	Exon 44	
			c.8875C>A	p.L2959M	Exon 44	[23]
			c.8921C>A	p.S2974X	Exon 44	[4]
			c.9272T>C	p.I3091T	Exon 44	[4, 23, 49]
GUCA1B	NM_002098	ad	c.469G>A	p.G157R	Exon 3	[4, 56]
IMPDH1	NM_001142573	ad	c.680T>C	p.L227P	Exon 7	[62]
			c.713A>G	p.K238R	Exon 7	[62]
			c.776G>A	p.R259H	Exon 7	[28]
IMPG2	NM_016247	ar	c.3262C>T	p.R1088X	Exon 16	[49]
JAG1	NM_000214	ad	c.1511A>G[e]	p.N504S	Exon 12	[49, 63]
LRAT	NM_004744	ar	c.163C>T	p.R55W	Exon 2	[49]
MAK	NM_001242385	ar	c.340dupG	p.A114Gfs	Exon 5	[49]
			c.496C>T	p.R166C	Exon 7	[49]
			c.553G>A	p.A185T	Exon 7	[49]
MERTK	NM_006343	ar	c.225delA	p.G76Efs	Exon 2	[49]
			c.370C>T	p.Q124X	Exon 2	[49]
			c.1450G>A	p.G484S	Exon 9	[49]
[a]*NR2E3*	NM_014249	ar	c.364C>T	p.R122C	Exon 4	[49]
[a]*NRL*	NM_006177	ad	c.23delT	p.L8Rfs	Exon 2	[49]

PDE6B	NM_000283	ar	c.993-1G>C		Intron 6	[49]
			c.1467+1G>C		Intron 11	[47, 49]
			c.1576G>A	p.E526K	Exon 12	[49]
			c.1604T>A	p.I535N	Exon 12	[49, 54]
			c.1669C>T	p.H557Y	Exon 13	[28, 49]
			c.1811C>T	p.T604I	Exon 14	[28]
			c.2012T>C	p.L671P	Exon 16	[28]
PRCD	NM_001077620	ar	c.2T>C	p.M1T	Exon 1	[49]
PRPF3	NM_004698	ad	c.1481C>T	p.T494M	Exon 11	[60]
PRPF6	NM_012469	ad	c.550G>C	p.D184H	Exon 5	[49]
PRPF31	NM_015629	ad	c.523delC	p.Q175Rfs	Exon 6	[4]
			c.528-3_528-45del		Intron 6	[55]
			c.562G>T	p.E188X	Exon 7	[49]
			c.613_615delTAC	p.Y205X	Exon 7	[4]
			c.615C>G	p.Y205X	Exon 7	[49]
			c.670G>A	p.G224R	Exon 7	[28]
			c.764A>T	p.Q255L	Exon 8	[49]
			c.1140_1141insTC	p.G381Sfs	Exon 11	[4]
			c.1142delG	p.G381Efs	Exon 11	[55]
			c.1155_1159delGGACG/insAGGGATT	p.D386Gfs	Exon 12	[55]
PRPH2	NM_000322	ad	c.410G>A	p.G137D	Exon 1	[28, 49]
			c.454A>G	p.M152V	Exon 1	[28]
			c.499G>A[f]	p.G167S	Exon 1	[28, 49, 59]
			c.589A>G	p.K197E	Exon 2	[28]
			c.629C>T	p.P210L	Exon 2	[9]
			c.641G>C	p.C214S	Exon 2	[52]
			c.732C>A	p.N244K	Exon 2	[33, 41, 42]
			c.736T>C	p.W246R	Exon 2	[49]
			c.811_813delCTC	p.L271del	Exon 2	[28]
			c.946T>G	p.W316G	Exon 3	[28]
RDH12	NM_152443	ad	c.377C>T	p.A126V	Exon 6	[49]
			c.776delG	p.E260Rfs	Exon 8	[49]

(*continued*)

Table 11.3 (continued)

Gene	Accession No.	Mode of inheritance	Nucleotide change	Predicted effect	Location in gene	References
[a]*RHO*	NM_000539	ad	c.36delC	p.P12Sfs	Exon 1	[4]
			c.44A>G	p.N15S	Exon 1	[18, 28, 49]
			c.50C>T	p.T17M	Exon 1	[17]
			c.180C>A	p.Y60X	Exon 1	[49]
			c.266G>A	p.G89D	Exon 1	[28]
			c.302G>A	p.G101E	Exon 1	[4]
			c.316G>A	p.G106R	Exon 1	[10]
			c.377G>T	p.W126L	Exon 2	[31]
			c.403C>T	p.R135W	Exon 2	[28, 49]
			c.520G>A	p.G174S	Exon 2	[28]
			c.541G>A	p.E181K	Exon 3	[53]
			c.562G>A	p.G188R	Exon 3	[49]
			c.977_980delACCC	p.P327Wfs	Exon 5	[49]
			c.979_982delCCAC	p.P327Wfs	Exon 5	[28]
			c.1036G>C	p.A346P	Exon 5	[31]
			c.1040C>T	p.P347L	Exon 5	
ROM1	NM_000327	ad	c.331dupG	p.L114Afs	Exon 1	[49]
[a]*RP1*	NM_006269	ar	c.649delG	p.G217Efs	Exon 3	[49]
			c.1186C>T	p.R396X	Exon 4	[49]
RP1L1	NM_178857	ar	c.235C>T	p.R79C	Exon 2	[49]
			c.1972C>T	p.R658X	Exon 4	[49]
RP2	NM_006915	X-linled	c.358C>T	p.R120X	Exon 2	[26, 37]
			c.758T>G	p.L253R	Exon 2	[61]
			c.832_833dupTC	p.Q278Lfs	Exon 3	[38]
[a]*RPE65*	NM_000329	ar	c.133T>C	p.C45R	Exon 3	[49]
			c.1543C>T[g]	p.R515W	Exon 14	[32, 34, 49]
RPGR	NM_001034853	X-linled	c.469+1G>A		Intron 5	[49]
			c.894_895delTT	p.S298Rfs	Exon 8	[49]
			c.922G>C	p.A308P	Exon 8	[49]
			c.1087_1088insGTAG	p.V363Gfs	Exon 10	[49]
			c.1981G>T	p.E661X	Exon 15	[49]
			c.2236_2237delGA	p.E746Rfs	Exon 15	[26]

			c.2321_2324delAGAG	p.E774Gfs	Exon 15	[28]
			c.2405_2406delAG	p.E802Gfs	Exon 15	[26]
			c.2426_2427delAG	p.E809Gfs	Exon 15	[26]
			c.2506_2507insG	p.E836Gfs	Exon 15	[66]
			c.2586_2587delGG	p.E863Rfs	Exon 15	[66]
			c.2614_2615insGG	p.E872Gfs	Exon 15	[66]
			c.1-80_1414+42delinsC		Exon1–11	[27]
SAG	NM_000541	ar	c.926delA[h]	p.N309Tfs	Exon 11	[16, 30, 44]
SNRNP200	NM_014014	ad	c.1871G>A	p.R624K	Exon 15	[49]
			c.2042G>A	p.R681H	Exon 16	[49]
			c.2047G>T	p.V683L	Exon 16	[49]
TOPORS	NM_005802	ad	c.2554_2557delGAGA	p.E852Qfs	Exon 3	[49]
TULP1	NM_003322	ar	c.G3A	p.M1I	Exon 1	[30]
			c.349G>A	p.E117K	Exon 4	[49]
			c.1145T>C	p.F382S	Exon 12	[34, 49]
			c.1246C>T	p.R416C	Exon 13	[30]
USH2A	NM_206933	ar	c.486-14G>A[i]		Intron 2	[35, 67]
			c.490G>T[i]	p.V164F	Exon 3	[49]
			c.685G>C	p.G229R	Exon 4	[67]
			c.1722_1723insGA	p.C575Dfs	Exon 10	[49]
			c.2776C>T	p.R926C	Exon 13	[67]
			c.3595_3597delGAA	p.E1199del	Exon 17	[67]
			c.6399G>A	p.W2133X	Exon 33	[49]
			c.7156G>T	p.V2386F	Exon 38	[67]
			c.7880T>C	p.I2627T	Exon 41	[30]
			c.8559-2A>G[j]		Intron 42	
			c.8682delG	p.R2894Sfs	Exon 44	[49]
			c.9676C>T	p.R3226X	Exon 49	[30]
			c.9751T>C[i]	p.C3251R	Exon 50	[3, 49]
			c.10544A>G[i]	p.D3515G	Exon 53	[39, 49]
			c.10859T>C	p.I3620T	Exon 55	[30]
			c.10931C>T	p.T3644M	Exon 55	[30]
			c.13466G>A	p.G4489D	Exon 63	[49]
			c.13847G>T	p.G4616V	Exon 64	[49]

(*continued*)

Table 11.3 (continued)

Gene	Accession No.	Mode of inheritance	Nucleotide change	Predicted effect	Location in gene	References
			c.14243C>T	p.S4748F	Exon 65	[49, 67]
			c.14450G>A	p.G4817E	Exon 66	[49]
			c.15178T>C	p.S5060P	Exon 70	[30]
			c.15233C>G	p.P5078R	Exon 70	[49]

Nucleotide numbering reflects cDNA numbering with +1 corresponding to A of the ATG translation initiation codon in the reference sequences
The initiation codon was designated as codon 1
Some DNA variations may be described using various mutation symbols (HGVS names). For example, the c.4957_4958insA mutation may be referred to as the c.4957dupA mutation
For convenience, we show only one symbol
[a]*BEST1*, *NR2E3*, *NRL*, *RHO*, *RP1*, and *RPE65* can cause both the ad and ar forms of RP (RetNet: https://sph.uth.edu/retnet/)
The following mutations have been reported as causes of not only RP but also other inherited retinal diseases
[b]Compound heterozygous mutations involving c.1760+2T>G or homozygous c.1760+2T>G mutations in *ABCA4* have been reported to cause Stargardt disease [19]
[c]Heterozygous mutation of c.763C>T in *BEST1* has been reported to cause best vitelliform macular dystrophy [65]
[d]Compound heterozygous c.4957_4958insA and c.8868C>A mutations in *EYS* have been reported to cause of ar cone-rod dystrophy [29]
[e]Heterozygous mutation of c.1511A>G in *JAG1* has been reported to cause Alagille syndrome [63]
[f]Heterozygous mutation of c.499G>A in *PRPH2* has been reported to cause pattern dystrophy [59]
[g]Homozygous mutations of c.1543C>T in *RPE65* have been reported to cause ar Leber congenital amaurosis [32]
[h]Homozygous mutations of c.926delA in *SAG* have been reported to cause Oguchi disease [16]
[i]Compound heterozygous mutations involving c.486-14G>A, c.490G>T, c.9751T>C, or c.10544A>G in *USH2A* have been reported to cause Usher syndrome type 2 [3, 35, 39, 49]
[j]Compound heterozygous mutations involving c.5886-2A>G or homozygous c.8559-2A>G mutations in *USH2A* have been reported to cause Usher syndrome type 2 [39, 40, 67]
ad autosomal dominant, *ar* autosomal recessive

according to several criteria for our own assessment [23, 67]. One of the common criteria for pathogenic mutations has been to determine its presence or absence in in-house normal control subjects. Several years ago, a variation was considered to be probably pathogenic based on its absence in 200 control chromosomes and more recently in larger numbers (400–1000 chromosomes). However, a recent study showed experimentally that 1 in 4–5 individual controls from the general world population may be a carrier of null mutations that are responsible for inherited retinal disease [46], suggesting that some of the rare variations identified as disease causing were present in the genomes of individual controls. Meanwhile, other studies reported that 27% of mutations cited in the literature were either common polymorphisms or lacked direct evidence for pathogenicity [8, 48]. Some of the publically reported mutations may turn out to be nonpathogenic in future studies. Indeed, Pozo MG et al. suggested that the p. C759F mutation in *USH2A*, which is frequently identified in Caucasian patients, might not be pathogenic [50].

Gene therapy seems promising for treating patients with inherited retinal diseases [6]. A more accurate molecular diagnosis is a prerequisite for selecting patients for clinical trials and is essential for determining potential therapeutic interventions, particularly for gene therapy. Therefore, based on current evidence, it is necessary to improve the assessment of variations associated with pathogenicity and to perform a careful reexamination of publically reported mutations.

Compliance with Ethical Requirements Katsuhiro Hosono, Shinsei Minoshima, and Yoshihiro Hotta declare that they have no conflict of interest.

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

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Cone Dysfunction Syndrome in the Japanese Population

12

Takaaki Hayashi

Abstract

Cone dysfunction syndromes (CDS) are a heterogeneous group of hereditary retinal disorders characterized by impaired visual acuity, defective color vision, nystagmus, and photophobia, which occur at birth or during early infancy. In contrast to the cone or cone-rod dystrophies, CDS are classified as stationary disorders, and which include congenital achromatopsia (ACHM, also known as rod monochromacy) with an autosomal recessive inheritance pattern. A total of six genes (*CNGA3*, *CNGB3*, *GNAT2*, *PDE6C*, *PDE6H*, and *ATF6*) have been identified as causes of ACHM. However, only the mutations *CNGA3*, *CNGB3*, and *PDE6C* have been identified in the few cases of achromatopsia that have been reported in Japanese patients. Thus, this chapter describes the detailed clinical and molecular genetic findings for ACHM in Japanese patients.

Keywords

Achromatopsia • Cone dysfunction • Hereditary retinal disorder • Autosomal recessive

12.1 Introduction

Cone dysfunction syndromes (CDS) are a heterogeneous group of hereditary retinal disorders characterized by impaired visual acuity, defective color vision, nystagmus, and photophobia which occur at birth or early infancy [1, 2]. In contrast to cone or cone-rod dystrophies, CDS are classified as stationary disorders, which include congenital achromatopsia (ACHM, also known as rod monochromacy) and blue-cone monochromatism (BCM) [1, 2]. To date, the six genes that have been identified as causes of ACHM include cyclic nucleotide-gated channel alpha-3 (*CNGA3*) [3], cyclic nucleotide-gated channel beta-3 (*CNGB3*) [4, 5], guanine nucleotide-binding protein, alpha-transducing

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activity polypeptide 2 (*GNAT2*) [6, 7], phosphodiesterase 6C (*PDE6C*) [8], phosphodiesterase 6H (*PDE6H*) [9], and activating transcription factor 6 (*ATF6*) [10, 11]. This chapter describes genotype-phenotype associations found in ACHM, especially in Japanese patients.

12.2 Epidemiology

ACHM is an autosomal recessive disorder with an estimated frequency that ranges from 1:30,000 to 1:50,000 in the European descent populations [1, 12]. However, little is known about the prevalence of ACHM in the Asian Pacific region, including Japan.

12.3 Clinical Features

ACHM is a congenital and basically stationary disorder characterized by low visual acuity, pendular nystagmus, photophobia, and severe color vision defects. Although the fundus appearance is usually normal, macular pigmentary changes and macular atrophy have been described. Visual field testing shows a small central scotoma in most patients. Full-field electroretinography (ERG) shows normal rod responses but severely reduced cone responses. Spectral-domain optical coherence tomography (SD-OCT) can detect an abnormal structure of the outer retinal layer including disruption of the inner segment ellipsoid line.

12.4 Molecular Genetic Aspects

To date, the six genes identified as the causes of ACHM include *CNGA3*, *CNGB3*, *GNAT2*, *PDE6C*, *PDE6H*, and *ATF6*. The proteins encoded by five of these genes (*CNGA3*, *CNGB3*, *GNAT2*, *PDE6C*, *PDE6H*) are exclusively expressed in the cone photoreceptors, where they are involved in the cone phototransduction cascade. A previous study found that *CNGB3* mutations account for half of the studied ACHM cases (48.2%), while *CNGA3* mutations account for 28.7%, *GNAT2* mutations account for 2.2%, and *PDE6C* mutations account for only 1.4% [13]. On the other hand, other studies [8, 14, 15] have reported that mutations in *CNGB3* account for 87% of the ACHM cases, with *CNGA3* accounting for 5%, and *PDE6C* for less than 1%. However, there were no mutations found for *GNAT2*. Out of the few cases of achromatopsia reported in Japan, only the *CNGA3*, *CNGB3*, and *PDE6C* mutations were identified in these Japanese patients [16–18]. Therefore, the mutation spectrum of ACHM in the Japanese population remains unknown.

12.5 Clinical Aspects (Case Studies)

This study examined two Japanese families with ACHM in whom the pathogenic mutations were identified.

12.6 Family 1

A 22-year-old female patient was referred for evaluation of photophobia and visual loss in both eyes [17]. Childhood history included nystagmus and low visual acuity. Decimal best-corrected visual acuity (BCVA) was 0.1 in both eyes, with refractions of −2.75 cylinder 10 deg. in the right eye and +1.25 sphere, −3.00 cylinder 270 deg. in the left eye. Ophthalmoscopy showed no specific findings in the optic disks or maculae (Fig. 12.1). Time-domain OCT assessment by retinal mapping disclosed a 20% thinner parafoveal thickness compared to a normal individual, even though the foveal thickness was normal (Fig. 12.1). Goldmann visual field tests showed bilateral 5-deg. central scotomas with the I-2e isopters, while the visual fields with the V-4e, I-4e, I-3e, I-2e, and I-2c targets were within normal limits in both eyes. This patient was only able to identify the first plate in the Ishihara test. The Farnsworth Panel D-15 showed there was confusion close to the scotopic axis. Although full-field ERG showed normal responses in the rod and maximal (mixed rod-plus-cone) responses, there was an extreme reduction in the cone and 30-Hz flicker responses (Fig. 12.2). Spectral sensitivity on a white

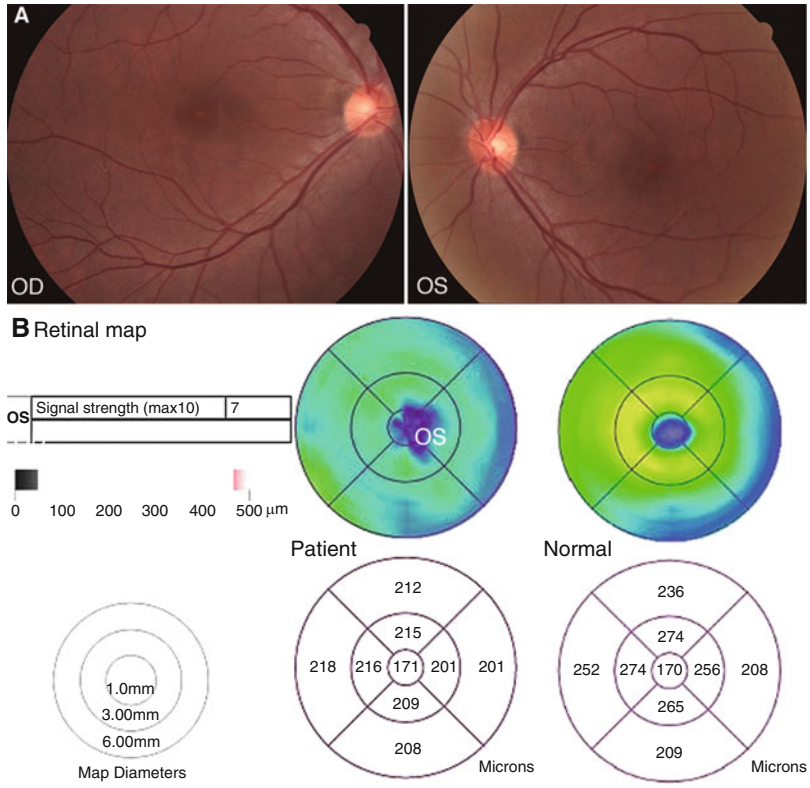

Fig. 12.1 Ophthalmoscopy in the patient of Family 1 found no specific findings in the optic disks or the maculae (**a**). Time-domain OCT assessment by retinal mapping disclosed a 20% thinner parafoveal thickness compared to a normal individual, even though the foveal thickness was normal (**b**)

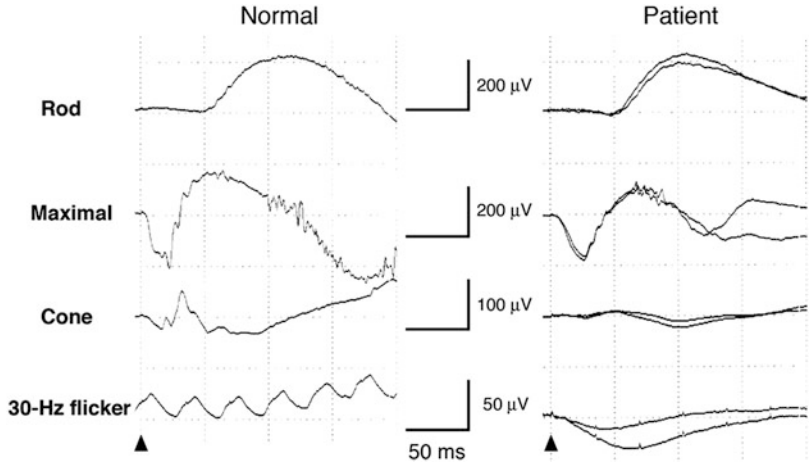

Fig. 12.2 Full-field electroretinography in the patient of Family 1 demonstrated there were normal responses for the rod and maximal (mixed rod-plus-cone) responses, but extremely reduced responses for the cone and 30-Hz flicker responses

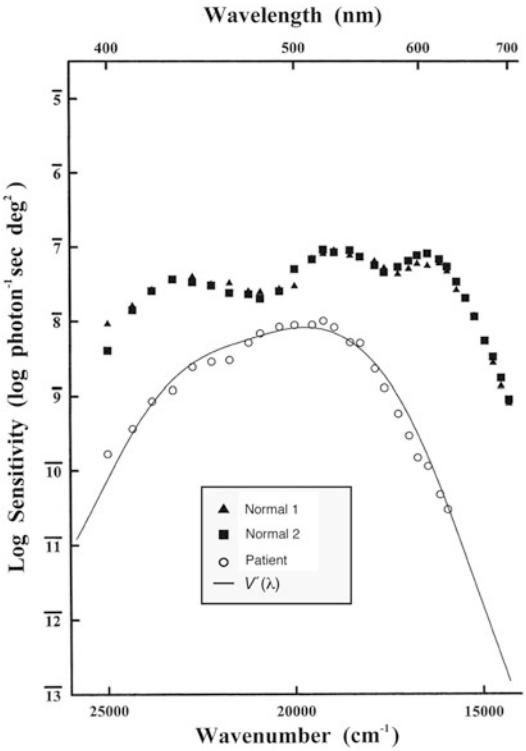

Fig. 12.3 Spectral sensitivity on a white background was measured in the patient of Family 1 and in two individuals with normal color vision using a three-channel Maxwellian view optical system. The two individuals with normal color vision showed curves with three peaks that consisted of short wavelength-sensitive (S), middle wavelength-sensitive (M), and long wavelength-sensitive (L) cones. However, the patient's curve revealed only one peak at around 500 nm, which is comparable to the spectral luminous efficiency function $V'(\lambda)$ for scotopic vision that fits the absorption spectrum of human rhodopsin

background was measured in the patient and in two individuals who exhibited normal color vision using a three-channel Maxwellian view optical system as previously described [19]. The spectral sensitivity measurements for the two individuals with normal color vision showed curves with three peaks that consisted of short wavelength-sensitive (S), middle wavelength-sensitive (M), and long wavelength-sensitive (L) cones. However, the patient's curve revealed only one peak at around 500 nm (Fig. 12.3), which was comparable to the spectral luminous efficiency function $V'(\lambda)$ for scotopic vision that fits the absorption spectrum of human rhodopsin. The patient's parents and sister reported no ocular complaints.

The mutation analysis using Sanger sequencing identified compound heterozygous *CNGA3* mutations (c.1306C>T; p. R436W, c.1898T>C; p. L633P) in the patient (Fig. 12.4) [17]. In the familial analysis, her father and sister carried the p. R436W mutation, while her mother carried the p. L633P mutation. The L633 is not only a phylogenetically conserved amino acid residue among mammalian orthologs, but it is also one of the most important hydrophobic residues in the CLZ domain downstream of the cGMP-binding site.

12.7 Family 2

A 7-year-old sister (patient 1) and her 5-year-old brother (patient 2) were referred to our department because of poor visual acuity [19]. Their parents had a consanguineous marriage. Since infancy, both patients have had hemeralopia, photophobia, and pendular nystagmus. At the time of their first visit to our hospital, the BCVA was OD, 0.1, and OS, 0.1; refractions were OD, +3.00, 0.5 X 180, and OS, +2.50, −0.75 X 180 (patient 1), and +7.00, −0.5 X 180 in both eyes (patient 2). Goldmann visual field testing that was done several years after their first visit showed there similar findings between the patients. Both patients 1 and 2 had bilateral relative central (5 deg) scotomas of the I-3c and I-2e isopters, respectively, while the peripheral visual fields with V-4e, III-4e, and I-4e isopters were normal. Both patients were only able to recognize the first plate in the Ishihara color vision test. The Farnsworth Panel D-15 indicated that both patients exhibited confusion close to a scotopic axis. The Nagel model I anomaloscope examination showed color matches along the rod line of the Rayleigh equation. Ophthalmoscopy showed that while patient 1 had atrophic-appearing macular lesions, patient 2 had no specific retinal findings. Full-field ERG showed normal rod and maximal responses, but there was an absent 30-Hz flicker response in both eyes. On the other hand, the cone ERGs differed, with the responses absent in patient

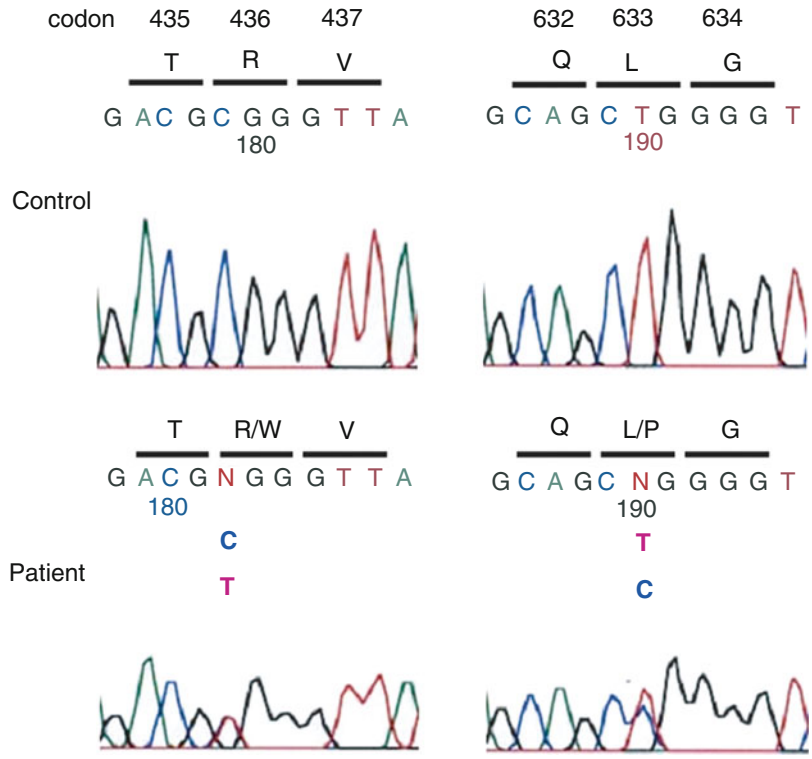

Fig. 12.4 Mutation analysis performed for the patient of Family 1 detected compound heterozygous *CNGA3* mutations (c.1306C>T; p. R436W, c.1898T>C; p. L633P)

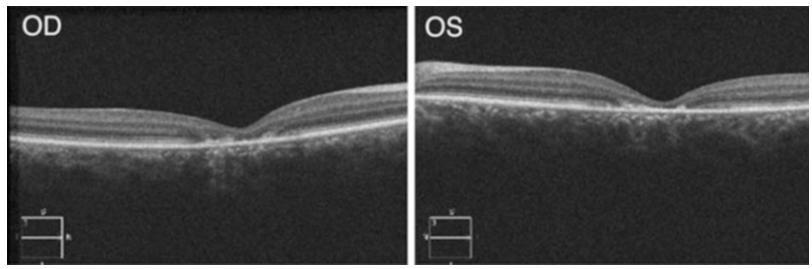

Fig. 12.5 Spectral-domain optical coherence tomography performed in patient 2 of Family 2 revealed retinal thinning with a loss of the outer retinal layer in each macula

1, while the amplitude of the a- and b-waves were reduced in patient 2. Therefore, patients 1 and 2 were diagnosed with complete and incomplete ACHM, respectively. Subsequently, both patients have undergone longitudinal follow-ups.

At the age of 30, the BCVA for patient 1 was 0.2 (with moderate myopia) in her right eye and 0.1 (with moderate myopia) in her left eye. Funduscopy showed atrophic chorioretinal macular scarring in the right eye and macular atrophy in the left eye. FAI revealed complete loss of autofluorescence in the macular lesions of both eyes. SD-OCT revealed severe macular thinning of all layers in both eyes and retinal and choroidal excavation in the right eye. Visual field testing showed there were bilateral central scotomas (5–10 deg) of the I-3e or I-2e isopters, even though the peripheral visual fields of the I-5e and I-4e isopters were normal. There was a slight broadening of the central scotomas relative to that seen at 11 years of age. At the age of 26, the BCVA for patient 2 was 0.2 (with high hyperopia) in each eye. Fundus autofluorescence imaging revealed hyper-autofluorescent areas of the maculae in both eyes. SD-OCT revealed

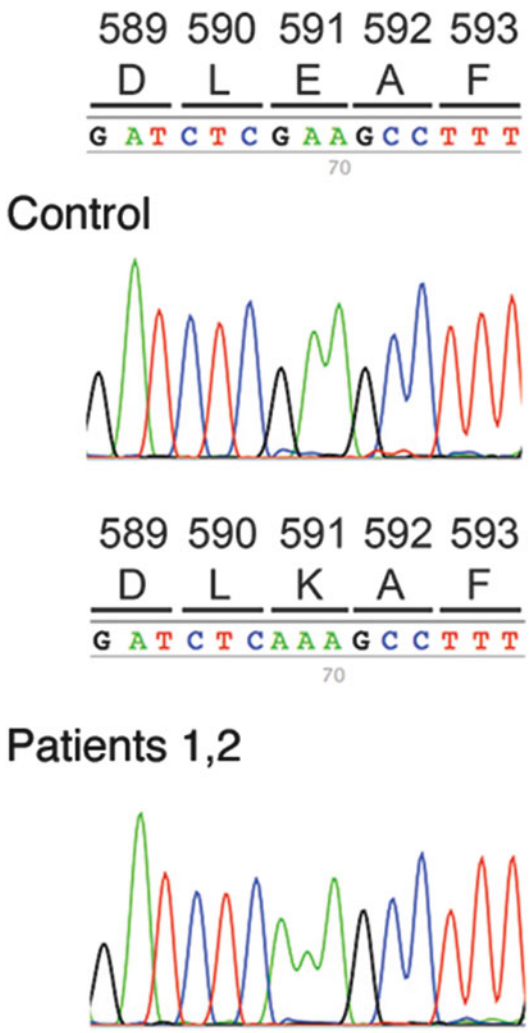

Fig. 12.6 A novel homozygous mutation (c.1771G>A, p. E591K) was identified in exon 14 of the *PDE6C* gene in patients 1 and 2 of Family 2

retinal thinning with loss of the outer retinal layer in each macula (Fig. 12.5).

In order to identify disease-causing variants in the mutation analysis, whole-exome sequencing was performed in each of the family members (patient 1, patient 2, and their parents). In each patient, we identified a novel homozygous mutation (c.1771G>A, p. E591K) in exon 14 of the *PDE6C* gene (Fig. 12.6) [18]. There were no pathogenic variants detected in the *CNGA3*, *CNGB3*, *GNAT2*, *PDE6H*, or *ATF6* genes. Interestingly, there was one known disease-causing mutation (p. G79R) in *OPN1SW,* which is associated with congenital tritan color vision deficiencies. The father and patient 1 each had the p. G79R mutation heterozygously, while the mother and patient 2 had neither mutation [18]. Since spectral ERG indicated that the father exhibited blue-yellow color vision deficiencies and no S-cone response, he was diagnosed with congenital tritan deficiencies [18]. The different phenotypes (complete and incomplete ACHM) between the patients might be explained by a "direct effect" or "possible modifier effect" related to the *OPN1SW* mutation (p. G79R) that was found in patient 1, who had complete ACHM.

12.8 Summary and Perspective

This chapter summarized the detailed clinical and molecular genetic findings for Japanese ACHM patients [17, 18]. However, there have been only three reports in which mutations (*CNGA3*, *CNGB3*, and *PDE6C*) were identified in Japanese ACHM patients [16–18]. Unlike ACHM patients of European descent, the mutation spectrum has yet to be clarified in the Japanese population. Therefore, a large cohort that investigates the genotype-phenotype correlations in ACHM in the Japanese population will need to be undertaken in the future.

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Leber Congenital Amaurosis/ Early-Onset Retinal Dystrophy in Japanese Population

13

Kazuki Kuniyoshi and Yoshikazu Shimomura

Abstract

The purpose of this chapter is to summarize the evidence on the correlations between the clinical features of Leber congenital amaurosis/early-onset retinal dystrophy (LCA/EORD) and the causative genes and to present the clinical characteristics of representative cases for each causative gene. Twenty-five genes are known causes of LCA/EORD: *GUCY2D*, *RPE65*, *SPATA7*, *AIPL1*, *LCA5*, *RPGRIP1*, *CRX*, *CRB1*, *NMNAT1*, *CEP290*, *IMPDH1*, *RD3*, *RDH12*, *LRAT*, *TULP1*, *KCNJ13*, *GDF6*, *PRPH2*, *OTX2*, *IQCB1*, *IFT140*, *PNPLA6*, *PEX1*, *CABP4*, and *MERTK*. Among these, the genes found in Japanese patients are *GUCY2D*, *RPE65*, *RPGRIP1*, *CRX*, *CRB1*, *NMNAT1*, *IMPDH1*, and *RDH12*. Most mutations discovered in Japanese patients have not been reported in other ethnic populations. Moreover, recent publications on genetic investigations in large cohort of Chinese patients with LCA/EORD revealed genetic characteristics different from those of the European/American populations. These results suggest that further genetic investigations on Japanese/Asian populations are needed.

Keywords

Leber congenital amaurosis • Early-onset retinal dystrophy • Japanese • Chinese • Korean • Asian • *GUCY2D* • *RPE65* • *SPATA7* • *AIPL1* • *LCA5* • *RPGRIP1* • *CRX* • *CRB1* • *NMNAT1* • *CEP290* • *IMPDH1* • *RD3* • *RDH12* • *LRAT* • *TULP1* • *KCNJ13* • *GDF6* • *PRPH2* • *OTX2* • *IQCB1* • *IFT140* • *PNPLA6* • *PEX1* • *CABP4* • *MERTK*

13.1 Introduction

Leber congenital amaurosis (LCA) is the most severe form of hereditary retinal dystrophy. Its worldwide prevalence is estimated to be 1 in

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30,000–80,000 births [24, 54, 102, 118]. The genotype of LCA is heterogeneous, and more than 20 genes have been identified as causative for this disease [22]. Thus, LCA is not a single entity but a group of retinal dystrophies. LCA is now divided into several subtypes, and the classification is based on the causative genes (Table 13.1).

13.1.1 Discovery of Leber Congenital Amaurosis (Leber [60, 61])

In 1869, Theodor Karl Gustav von Leber (1840-1917) reported on infants who had severe nystagmus and sluggish pupillary light reflexes. Some of them were completely blind and others had photophobia. Their fundi were normal in the first month of life; however the fundi progressed to pigmentary retinal degeneration within several years accompanied by a marked decrease of vision. Some of the less severely affected children had difficulty in dark environments, i.e., night blindness. He described these infants as "intrauterine retinitis pigmentosa (RP)." He believed that this disease was an inherited disorder because their parents were often consanguineous and their siblings were often blind. This condition is now recognized as Leber congenital amaurosis (LCA, MIM# 204000). Leber [62] also recognized that the phenotype of LCA was heterogeneous and early-onset retinal dystrophy (EORD) were diseases allied to the congenital amaurosis. One hundred years later, these insights of Leber were confirmed at the molecular level. Thus at present, patients with these phenotypes are referred to as LCA/EORD cases.

13.1.2 Symptoms and Diagnosis of LCA

The diagnosis of LCA is usually made before 1 year of age and classically before 1 month. The infants with LCA present with severe visual impairments (no pursuit of objects), searching nystagmus, and absent or sluggish pupillary light reflexes. Their fundi can be normal or abnormal. The presence of the digito-ocular sign (eye pressing, eye rubbing, and eye poking) is a manifestation of LCA [33]. Non-recordable (extinguished) or severely reduced electroretinograms (ERGs) are the bases for diagnosis of LCA [32], because this is a congenital disorder of photoreceptors. Other congenital disorders that show poor vision and nystagmus can be differently diagnosed by the ERG findings (see the "infantile nystagmus syndrome").

With increasing age, the fundi of LCA patients can progress from normal to different degrees of retinal degeneration including a flecked retina, diffuse pigmentary retinal degeneration, and coloboma-like macular atrophy/posterior staphyloma and other alterations. In addition, keratoconus and cataract can be present in some of these patients.

The behavior of patients with LCA can be divided into two types: photophobia and night blindness. Perrault et al. [83] and Hanein et al. [36] referred to them as Type 1 and Type 2, respectively, and they summarized the characteristics of these types as Type 1, severe and stationary cone-rod dysfunction with hyperopia and vision worse than hand motion, and Type 2, rod-cone dystrophy with variable refractive errors and vision usually better than hand motion.

This subclassification is clinically useful, although at the present time, it is not always applicable to every patient with LCA because of the increase in the number of causative genes for LCA/EORD.

13.1.3 LCA vs Infantile Nystagmus Syndrome

The infantile nystagmus syndrome should be ruled out in patients suspected of having LCA-like disease, namely, albinism, complete achromatopsia, blue cone monochromacy, and congenital stationary night blindness. They can

Table 13.1 Subtypes of Leber congenital amaurosis/early-onset retinal dystrophy (LCA/EORD)

LCA#	Inheritance	Prevalence		Symbol	Gene name	Gene function	Predominant dysfunction	Fundus	Other findings	Other diseases	Reports in Japan
		Caucasian	Chinese								
LCA1	AR	6–20%	12%	*GUCY2D*	Guanylate cyclase 2D	Recovery to dark state in cones	Cone	Normal to diffuse retinal degeneration	–	CORD6	Hosono et al. [40]
LCA2	AR	6–16%	4–5%	*RPE65*	Retinal pigment epithelium-specific protein 65 kDa	Retinoid cycle	Rod	Translucent RPE White dots Peculiar star-shaped maculopathy	Transient improvement of vision	RP20	Wada et al. [110] and Katagiri et al. [47]
LCA3	AR	1–5%	3%	*SPATA7*	Spermatogenesis-associated protein 7	Unknown	Rod>Cone	Peripheral retinal degeneration	–	Juvenile RP	None
LCA4	AR	4–8%	2–3%	*AIPL1*	Arylhydrocarbon-interacting receptor protein-like 1	Chaperone for cGMP PDE in cone/rod	Cone>Rod	Maculopathy and retinal degeneration	Keratoconus	Juvenile RP CORD	None
LCA5	AR	1–2%	3%	*LCA5*	Leber congenital amaurosis 5	Ciliary transportation between IS and OS of photoreceptors	Rod>Cone	Maculopathy and retinal degeneration	–	None	None
LCA6	AR	5%	7%	*RPGRIP1*	Retinitis pigmentosa GTPase regulator-interacting protein 1	Photoreceptor OS disc formation	Cone>Rod	Relatively mild	–	CORD13	Suzuki et al. [104]
LCA7	AD	Less than 3%	2–3%	*CRX*	Cone-rod homeobox	Differentiation of photoreceptors with *OTX2*, *NRL*, *TR-β2*, etc.	Rod>Cone	Macular coloboma and retinal degeneration	Transient improvement of vision	CORD2 MD	Nakamura et al. [75]
LCA8	AR	8%	13%	*CRB1*	Crumbs homolog 1	Polarity control of photoreceptors	Rod>Cone	RPE depigmentation, thickened retina Para-arteriolar RPE preservation	Keratoconus	RP12 Coats like PPCRA	Kuniyoshi et al. [58]

(continued)

Table 13.1 (continued)

LCA#	Inheritance	Prevalence		Symbol	Gene name	Gene function	Predominant dysfunction	Fundus	Other findings	Other diseases	Reports in Japan
		Caucasian	Chinese								
LCA9	AR	Unknown	1%	*NMNAT1*	Nicotinamide nucleotide adenylyltransferase 1	Neuroprotection	Rod>Cone	Macular coloboma	Non-syndromic	None	Coppieters et al. [20]
LCA10	AR	22%	6–7%	*CEP290 (NPHP6)*	Centrosomal protein 290 kDa (Nephronophthisis 6)	Ciliary transportation between IS and OS of photoreceptors	Cone>Rod	Normal to diffuse retinal degeneration	Renal failure Mental retardation Hypotonia	JBTS5 SLSN6 MKS4	None
LCA11	AD	Unknown	0.4%	*IMPDH1*	Inosine 5′-monophosphate dehydrogenase 1	Synthesis of guanine (housekeeping gene)	Rod>Cone	RPE mottling	Development delay Hypotonia	RP10	Wada et al. (juvenile RP) [111], [112]
LCA12	AR	1%	1%	*RD3*	Retinal degeneration 3	Accessory protein for GUCY2D (cone) and GUCY2F (rod)	Cone >Rod	Macular/retinal degeneration	–	None	None
LCA13	AR	4%	3%	*RDH12*	Retinol dehydrogenase 12	Retinoid cycle	Rod>Cone	Macular atrophy & retinal degeneration	Transient improvement of vision Cataract	RP53	Kuniyoshi et al. [57]
LCA14	AR	1%	Not found	*LRAT*	Lecithin retinol acyltransferase	Retinoid cycle	Rod>Cone	Retinal degeneration	Similar to LCA2	Juvenile RP	None
LCA15	AR	1%	2%	*TULP1*	Tubby-like protein 1	RPE phagocytosis	Rod>Cone	Retinal degeneration	–	RP14 COD	None
LCA16	AR	Unknown	Not found	*KCNJ13*	Potassium channel, inwardly rectifying subfamily J, member 13 (Kir7.1)	Control of inward flow of potassium surrounding RPE	Rod>Cone	Retinal degeneration	–	Snowflake vitreoretinal degeneration	None
LCA17	AR	Unknown	Not found	*GDF6*	Growth differentiation factor 6	Development of the eye	?	Not shown	–	KFS1 MCOPCB6 MCOP4	None

LCA18	AR	Unknown	Not found	*PRPH2 (RDS)*	Peripherin 2 (retinal degeneration slow)	Stabilization of outer segment of photoreceptors	?	Maculopathy and retinal degeneration	–	RP7 ARD VMD3 CACD2 MDPT1	None
–	AD	Unknown	0.4%	*OTX2*	Orthodenticle homeobox 2	Differentiation of photoreceptors with *CRX, NRL, TR-β2*, etc.	Rod>Cone	Granular pigmentation of the RPE	Negative ERG Non-recordable rod ERG Development delay	MCOPS5 CPHD6	None
–	AR	Unknown	1%	*IQCB1 (NPHP5)*	IQ motif-containing protein B1 (nephronophthisis 5)	Ciliary transportation between IS and OS of photoreceptors	Rod>Cone	Retinal degeneration	Renal failure	SLSN5	None
–	AR	Unknown	Not found	*IFT140*	Intraflagellar transport 140	Ciliary transportation between IS and OS of photoreceptors	?	Mottling and depigmented spots in RPE	Bone abnormality	Ciliopathy SRTD9	None
–	AR	Unknown	Not found	*PNPLA6 (NTE)*	Patatin-like phospholipase domain-containing protein 6 (neuropathy target esterase)	Membrane lipid homeostasis	?	Severe retinal degeneration	Autism	OMCS BNHS SPG39 LNMS	None
–	AR	Unknown	Not found	*PEX1*	Peroxisomal biogenesis factor 1	Assist the biogenesis of peroxisomes	?	Salt and pepper	Psychomotor retardation	Zellweger syndrome NALD/IRD	None
–	AR	Unknown	Not found	*CABP4*	Calcium-binding protein 4	Maintenance of synaptic function	Cone>Rod	Normal	Negative ERG Normal rod ERG	CSNB2B	None

(continued)

Table 13.1 (continued)

LCA#	Inheritance	Prevalence		Symbol	Gene name	Gene function	Predominant dysfunction	Fundus	Other findings	Other diseases	Reports in Japan
		Caucasian	Chinese								
–	AR	Unknown	1%	*MERTK*	MER tyrosine kinase proto-oncogene	RPE phagocytosis	Rod>Cone	Macular/retinal degeneration	–	RP38	None

Prevalence in the Chinese population was reported by Li et al. [64], Chen et al. [17], and Wang et al. [114]. *CORD* cone-rod dystrophy, *RP* retinitis pigmentosa, *MD* macular degeneration, *PPCRA* pigmented paravenous chorioretinal atrophy, *JBTS* Joubert syndrome, *SLSN* Senior-Løken syndrome, *MKS* Meckel syndrome, *COD* cone dysfunction, *KFS* Klippel-Feil syndrome, *MCOPCB* microphthalmia with coloboma, *MCOP* isolated microphthalmia, *ARD* albipunctate retinal dystrophy, *VMD* vitelliform macular dystrophy, *CACD* central areolar choroidal dystrophy, *MDPT* patterned macular dystrophy, *MCOPS* syndromic microphthalmia, *CPHD* combined pituitary hormone deficiency, *SRTD* short-rib thoracic dysplasia, *OMCS* Oliver-McFarlane syndrome, *BNHS* Boucher-Neuhauser syndrome, *SPG* spastic paraplegia, *LNMS* Laurence-Moon syndrome, *NALD/IRD* neonatal adrenoleukodystrophy/infantile Refsum disease, *CSNB* congenital stationary night blindness

be differentiated from LCA based on their characteristic ERG abnormalities [73, 116]. Albinism has normal or negative-type flash ERG; achromatopsia or blue cone monochromacy is characterized by normal or subnormal flash ERGs and non-recordable cone ERGs. Congenital stationary night blindness has the negative-type flash ERG. Therefore, non-recordable flash ERG is a unique and as important finding for the diagnosis of LCA.

13.1.4 Genetic Aspects and Gene Therapy on Patients with LCA/EORD

The majority of LCA/EORD cases have an autosomal recessive inheritance pattern. To date, almost 25 causative genes have been identified for LCA/EORD (LCA1–18 and others, Table 13.1; [22]). Among them, LCA1, 2, 6, 7, 8, 9, 11, and 13 have been reported in the Japanese population up to October 2015.

Recent discoveries of new causative genes for LCA have extended the clinical entities of LCA. Some of the mutations also cause juvenile- or even adult-onset retinal dystrophies.

Most of the causative genes for LCA/EORD are involved in the development and differentiation, protection and maintenance, and metabolism and function of the photoreceptors or retinal pigment epithelium (RPE). A schema of the visual transduction cascades and retinoid cycle and their related genes are shown in Fig. 13.1.

LCA/EORD is one of the best disorders for gene supplementation therapy because it is a disease of infants with profound visual impairment. In addition, most of the cases have an autosomal recessive inheritance pattern. Recently, gene supplementation therapies on patients with *RPE65*-associated LCA (LCA2) were reported to be successful by several groups [8, 70, 107]. However, there are still some problems that need to be solved [9, 45].

Genetic investigations of patients with LCA/EORD are very important. Genetic information will allow clinicians, patients, and their

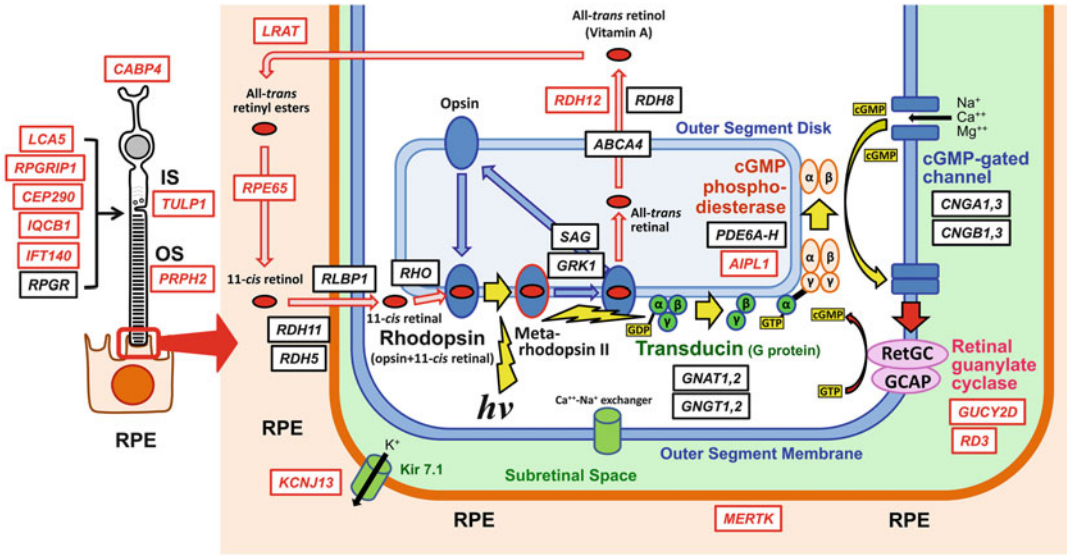

Fig. 13.1 Schematic diagram of the retinoid cycle (*pink arrows*), rhodopsin cycle (*light blue arrows*), visual phototransduction cascades (*yellow arrows*), and the genes associated with different steps in the cascades [29, 46, 63, 95]

Right diagram is a magnified image of the region of the *red square* in the *left* scheme. Gene symbols are indicated in *italics* with *squares*. *Red symbols* are causative genes for Leber congenital amaurosis/early-onset retinal dystrophy. *IS* inner segment of the photoreceptor, *OS* outer segment of the photoreceptor, *RPE* retinal pigment epithelium

1. *LCA5*, *RPGRIP1*, *CEP290*, *IQCB1*, *IFT140*, and *RPGR* in the *left* scheme are the genes associated with the cilia which bridge the inner and outer segment of the photoreceptors. The cilia play important roles in the transport of molecules between the inner and outer segments of the photoreceptors and in disk formation. *CABP4* is involved in signal transmission between photoreceptor and bipolar cells

2. *Yellow arrows* indicate phototransduction cascades. Light (photons, *hv*) photoactivates rhodopsin which results in a conformational change to metarhodopsin II which then initiates the phototransduction cascades and involves transducin and cGMP phosphodiesterase (PDE). Metarhodopsin II activates the transducin, which is a trimer, consisting α-, β-, and γ-subunits. The α-subunit of transducin is activated releasing GDP in exchange for cytoplasmic GTP, which expels the β- and γ-subunits. Next, the activated α-subunit of transducin binds the inhibitory γ-subunit of cGMP phosphodiesterase (PDE) activating α- and β-subunits of the PDE. Activated PDE breaks down cGMP to 5′-GMP that leads to the reduction of cGMP concentration in the photoreceptors. Then the cGMP-gated (CNG) channels close resulting in hyperpolarization of the photoreceptors. Thus, the light photons are converted to an electrical neural signal

3. The hyperpolarization of the photoreceptors closes voltage-gated Ca^{++} channels resulting in a decrease of concentration of Ca^{++} in the photoreceptors. The decrease of the intra-photoreceptor Ca^{++} also causes a decrease in the amount of glutamate which is released by photoreceptors as a signal transmitter. A decrease of the glutamate depolarizes the on-bipolar cells and hyperpolarizes the off-bipolar cells

4. *Red arrows* indicate recovery to dark-adapted state system. Then the decrease of intra-photoreceptor Ca^{++} activates the guanylate cyclase-activating protein (GCAP), a Ca^{++}-binding protein, and retinal guanylate cyclase (GUCY). GUCY begins converting GTP to cGMP, and then the photoreceptors return to their dark state after the light exposure

5. *Pink arrows* indicate retinoid cycle. The activated metarhodopsin II releases all-*trans* retinal. The all-*trans* retinal is isomerized to 11-*cis* retinal again through several steps and enzymes. Finally, rhodopsin is reproduced after the 11-*cis* retinal is bound into the opsin

6. *Light blue arrows* indicate rhodopsin cycle. Rhodopsin is photostimulated into metarhodopsin II via photo-, batho-, and lumi-rhodopsin (not shown). While the metarhodopsin II initiates the phototransduction cascades, it is inactivated by *GPK1* (G protein-coupled receptor kinase 1, rhodopsin kinase) and *SAG* (S-antigen, arrestin). Then metarhodopsin II releases all-*trans* retinal and remained opsin binds 11-*cis* retinal again, regenerating the rhodopsin

parents know the prognosis of the disease, matters to be attended to in the patient's daily life, and the chances of therapeutic interventions. Therefore, every clinician who sees patients with LCA/EORD is encouraged to investigate their genotype. The costs for gene investigation of the patients are covered by government or medical insurance in some European/American countries; however in Japan, it is usually covered by investigator's personal research funds. This is the reason why longitudinal genetic studies with large cohorts are difficult to be realized in Japan.

In this chapter, the genotype and function of the causative genes and phenotypes of each subtype of LCA/EORD are summarized. The clinical reports on Japanese patients with LCA/EORD are emphasized, and reports on the Asian population are also highlighted.

13.2 LCA1

LCA1 is caused by a homozygous mutation or compound heterozygous mutations in the guanylate cyclase 2D (*GUCY2D*) gene. LCA1 is one of the most frequently detected types of LCA/EORD with a prevalence of 6–20%. [24, 105, 118]. This gene is also known to be causative for autosomal dominant cone-rod dystrophy (CORD6) and autosomal recessive cone-rod dystrophy.

13.2.1 Function of *GUCY2D* Gene

The *GUCY2D* gene is located on the short arm of chromosome 17 at locus 13.1.

It encodes for the retinal guanylate cyclase 2D protein (RetGC1). RetGC1 is expressed in the outer segments of cone photoreceptors and plays an essential role in the recovery of the photoreceptors from the light-adapted to the dark-adapted state (Fig. 13.1). Stimulation of photoreceptor cells results in a reduction of intracellular Ca^{++} in the outer segments, and the reduction of intracellular Ca^{++} activates RetGC1 in cones and RetGC2 in rods. The activated RetGC opens the cyclic nucleotide-gated (CNG) channels (cGMP-gated channels, Fig. 13.1) which allow Ca^{++} to enter the outer segments again. This results in the recovery of the photoreceptors to the dark-adapted state [13, 90].

13.2.2 Clinical Features of LCA1

LCA1 is typical of Type 1 LCA. Patients with LCA1 are characterized by severe congenital cone-rod dystrophy, namely poor vision (worse than hand motion), photophobia, and high hyperopia. The fundus findings in infantile patients with LCA1 are mild or unremarkable [13]. The progression of the visual impairment is usually slow [36, 82]. Perrault et al. [85] reported on a patient with early-onset RP with homozygous mutation in the *GUCY2D* gene.

Milam et al. [72] reported on the histopathological findings of the retina of an 11-year-old patient with LCA1. Although the patient had visual acuity of only light perception, a substantial number of cones and rods were present not only in the macula but also in the far periphery. The authors concluded that these findings suggested a possibility for therapy of this disease.

13.2.3 LCA1 in Japanese/Asian Populations

Hosono et al. [40] reported on twin Japanese boys with LCA1 who had the typical phenotype of LCA1 (Type 1 LCA). They were noted to lack ocular pursuit movements at the age of 3 months. Fundus examinations performed at age 11 months revealed almost normal fundi except for mild RPE changes and slightly attenuated vessels. Both boys had the digito-ocular signs, nystagmus, and photophobia. The refractive errors were hyperopia, and the ERGs were non-recordable. Genetic investigations revealed a compound heterozygous splicing mutation

c.2113+2_2113+3insT and a missense mutation p.L905P in the *GUCY2D* gene in both twins. Both of these mutations were novel.

The results of recent comprehensive genetic investigations on patients with LCA/EORD were reported from China [17, 64, 114]. Their reports indicated that the prevalence of LCA1 in all Chinese patients with LCA/EORD was 12%. This suggests that *GUCY2D* is one of the most common causative genes for LCA in the Asian population.

13.3 LCA2

LCA2 is caused by a homozygous mutation or compound heterozygous mutations in the retinal pigment epithelium-specific protein 65 kDa (*RPE65*) gene. LCA2 accounts for 6–16% of all LCA cases [24, 105, 118]. This gene is also causative for autosomal recessive RP (RP20).

13.3.1 Function of *RPE65* Gene

The *RPE65* gene is located on the short arm of chromosome 1 at locus 31. The encoded RPE65 protein, retinoid isomerase, is synthesized by the RPE cells and helps isomerize all-*trans* retinyl ester back to 11-*cis* retinol in the retinoid cycle after exposure of the light (Fig. 13.1). Dysfunction of the RPE65 protein results in blocking the visual cycle more severely in rods than cones because the cone photoreceptors have an alternative pathway [106].

13.3.2 Clinical Features of LCA2

LCA2 has a typical Type 2 LCA phenotype. Patients with LCA2 complain of night blindness rather than photophobia because a dysfunction of RPE65 affects the rods more than cones. Their visual functions can have transient improvements in the early stage of the disease [83]. The phenotype of LCA2 appears to be milder than that in the other LCAs and is characterized by less pigmentary changes in the retina, translucent RPE, white dots, and a peculiar star-shaped maculopathy [117].

Weleber et al. [117] reported on the clinical findings of five patients with *RPE65*-associated LCA/EORD. All had night blindness from birth. Their decimal best-corrected visual acuity (BCVA) was between 0.05 and 0.1, and the BCVA was relatively well maintained up to 3 to 18 years of age. The rod ERGs were non-recordable whereas the cone ERGs were recordable. One patient had an improvement of the ERG amplitude including the rod ERGs between the ages of 10 and 14 years. The fundus of these patients had a mottled RPE appearance; however pigmentations were rare. White deposits were observed in two patients.

13.3.3 Gene Therapy for LCA2: Highlights of LCA2 Gene Therapy Trials

In 2008, Maguire et al. [70] and Bainbridge et al. [8] reported on the short-term results of gene therapy in patients with LCA2. A recombinant adeno-associated virus (AAV) carrying *RPE65* complementary DNA was injected into the subretinal space of six patients with LCA2. Each of the three patients in study by Maguire el al. had a modest improvement in pupillary light reflexes and visual acuity. One patient in study by Bainbridge et al. had a significant improvement in the visual function on microperimetry and on dark-adapted perimetry; however the other two patients had no significant changes in the visual acuity. No adverse side effect was observed except one patient who developed an asymptomatic macular hole.

Testa et al. [107] reported their 3-year results of gene supplementation therapy on five patients with LCA2. They reported that the retinal sensitivity and BCVA improved after a subretinal injection of DNA, and the sensitivities and BCVAs have remain stable for at least 3 years after the injection. On the other hand, Bainbridge et al. [9] and Jacobson et al. [45] reported that the retinal function declined 3 or 6 years after the DNA injection. Bainbridge et al. concluded that

the amount of RPE65 required for human patients with LCA2 needs to be evaluated.

13.3.4 LCA2 in Japanese/Asian Populations

In Japan, four patients with LCA2 have been reported. Wada et al. [110] reported on two siblings with LCA2 who had compound heterozygous mutations of p.L450R and p.R515W in the *RPE65* gene. Detailed clinical findings were not reported.

Katagiri et al. [47] reported on two unrelated patients: one with compound heterozygous mutations of p.H59Q and p.D62X, these were novel, and the second with homozygous p.R515W mutations in the *RPE65* gene. The first patient was a young girl who was noticed to have nystagmus at 1 month of age. Fundus examinations at 5 months revealed a normal foveal reflex with mottled RPE in the periphery (Fig. 13.2). The ERGs were non-recordable, and she was diagnosed with LCA. At age 11 years, her BCVA was 0.08 in the right eye and 0.09 in the left eye. Both eyes were emmetropic but she had astigmatism of two to three diopters. The other patient was a 30-year-old woman. Her parents reported that she had night blindness and poor vision since the first year of life. Her BCVA was 0.06 in the right eye and 0.08 in the left eye, and both were emmetropic. Nystagmus was not remarkable. Fundus examination showed severe and diffuse retinal degeneration with macular atrophy in both eyes (Fig. 13.2). The full-field ERGs were non-recordable. Goldmann kinetic perimetry showed constricted visual fields with central scotoma in both eyes.

In Korea, one LCA2 patient was reported by Seong et al. [98].

The results of comprehensive genetic investigations on Chinese patients with LCA/EORD showed that the prevalence of LCA2 was 4–5%, which was lower than that in the Caucasian population [17, 64, 114].

13.4 LCA3

LCA3 is caused by a homozygous mutation or compound heterozygous mutations in the spermatogenesis-associated protein 7 (*SPATA7*) gene. The LCA3 cases are estimated to account for only 1–5% of all LCA/EORD cases, and therefore *SPATA7* is a rare cause of LCA/EORD [24, 105, 118]. This gene also causes autosomal recessive juvenile RP.

13.4.1 Function of *SPATA7* Gene

SPATA7 is located on the long arm of chromosome 14 at locus 31.3 and is expressed in the retina but was originally isolated from the testis. Although detailed information on the function of this gene has not been determined, *SPATA7* appears to be important for normal retinal function rather than retinal development. This is believed because it is not expressed in the early and middle developmental stage of mice retinas but is expressed in the fully developed retina [113].

13.4.2 Clinical Features of LCA3

The LCA associated with *SPATA7* mutations resembles infantile-onset severe rod-cone dystrophy with peripheral retinal degeneration and relatively well-preserved foveal structure (Type 2 LCA).

Wang et al. [113] reported on seven patients with *SPATA7*-associated LCA. Three patients in a consanguineous Saudi-Arabian family had poor visual fixation from birth and subsequently developed nystagmus and non-recordable ERGs. A Dutch girl who had visual problems since birth had peripheral chorioretinal atrophy at age 6 years. In contrast, a Portuguese girl who had nyctalopia since age 2 years had constricted (5 degrees) visual field, excellent BCVA (1.0), and no nystagmus. Her fundus had no

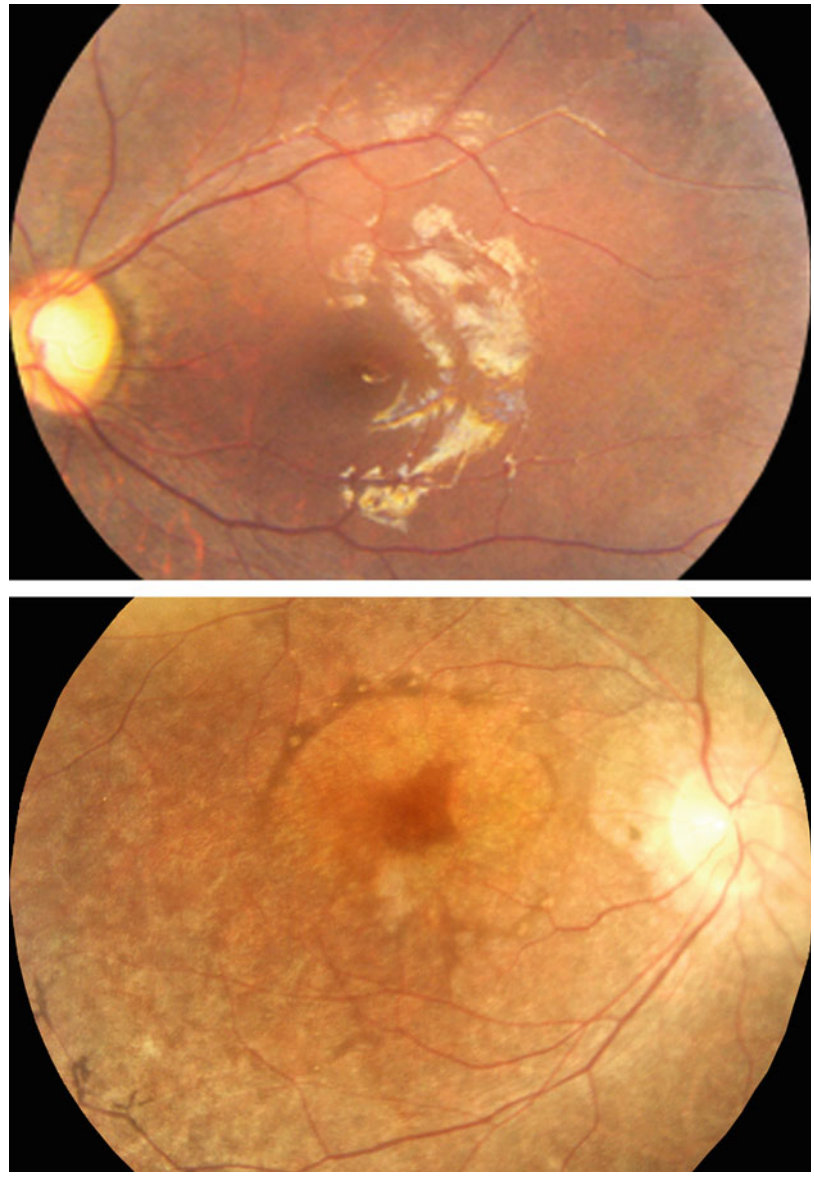

Fig. 13.2 Fundus photographs of Japanese patients with *RPE65* mutations (LCA2) These patients were a 5-year-old girl (*upper*) and a 30-year-old woman (*lower*) who were reported by Katagiri et al. in 2016 [47]. Their decimal best-corrected visual acuities (BCVAs) were 0.09 and 0.06, respectively. These photographs were provided by Dr. Takaaki Hayashi and Dr. Mineo Kondo

pigmentary changes although the retinal arteries were attenuated and a hypopigmented perifoveal anulus was present.

13.4.3 LCA3 in Japanese/Asian Populations

No LCA3 patient with *SPATA7*-associated retinopathy has been reported in the Japanese population. Results of comprehensive genetic investigations on Chinese patients with LCA/EORD indicated prevalence of LCA3 was 3%, which appears to be almost the same as that in the Caucasian population [17, 64, 114].

13.5 LCA4

LCA4 is caused by a homozygous mutation or compound heterozygous mutations of the arylhydrocarbon-interacting receptor protein-

like 1 (*AIPL1*) gene. LCA4 cases are estimated to account for 4–8% of all LCAs [24, 105, 118]. The *AIPL1* gene is also causative for juvenile RP and cone-rod dystrophy, both of which have an autosomal recessive inheritance pattern.

13.5.1 Function of *AIPL1* Gene

This gene is located on the short arm of chromosome 17 at locus 13.2. The AIPL1 protein is a member of the FK-506-binding protein family and is expressed specifically in the photoreceptor cells. This protein is thought to be a specialized chaperone required for cyclic guanosine monophosphate (cGMP) phosphodiesterase (PDE) biosynthesis both in cone and rod photoreceptors (Fig. 13.1) [51, 65].

13.5.2 Clinical Features of LCA4

The phenotype of LCA4 is a relatively severe maculopathy and is characterized by bone-spicule pigmented retinal degeneration. Sohocki et al. [101] first reported on five families with *AIPL1*-associated LCA. Two of them were blind from birth, and the others had poor central vision with severe night blindness from birth. The affected patients had diffuse pigmentary retinal degeneration with macular degeneration. The ERGs were non-recordable. The affected patients in a family with LCA4 had bilateral keratoconus.

Aboshiha et al. [1] reported on the fundus findings of 42 patients with LCA4; 18% were normal, 46% had retinal pigmentary changes without macular atrophy, and 33% had macular atrophy.

13.5.3 LCA4 in Japanese/Asian Populations

No LCA4 patient has been reported in the Japanese population. Results of comprehensive genetic investigations on Chinese patients with LCA/EORD indicated prevalence of LCA2 was 2–3%; it seems to be lower than that in the Caucasian population [17, 64, 114].

13.6 LCA5

LCA5 is caused by a homozygous mutation or compound heterozygous mutations in the Leber congenital amaurosis 5 (*LCA5*) gene. This gene is a relatively rare cause of LCA, and LCA5 cases account for only 1–2% of all LCAs [24, 105, 118]. However, it may account for 7–8% in the Spanish population [21].

13.6.1 Function of *LCA5* Gene

The *LCA5* gene is located on the long arm of chromosome 6 at locus 14.1. This gene encodes a protein called lebercilin which is expressed in the ciliary axoneme (microtubules) in human tissues. It is believed to be involved in the minus end-directed microtubule transportation. In photoreceptors, lebercilin is located in the cilia that bridge the inner and outer segments and plays an important role in the transportation of proteins between them (Fig. 13.1) [11].

13.6.2 Clinical Features of LCA5

Dharmaraj et al. [26] studied a consanguineous family belonging to the Old Order River Brethren in Pennsylvania in which three individuals were affected by LCA5. These patients had the typical phenotype of LCA, namely, poor vision, nystagmus, high hyperopia, and poor pupillary reflexes. Their fundi were initially unremarkable; however with increasing age, a peripheral retinal mottling of varying degree developed.

Corton et al. [21] reported a relatively high prevalence of LCA5 in the Spanish population. Their patients had low vision since birth or before the age of 1 year, and had nystagmus, night blindness, and visual field constriction (Type 2 LCA). The ERGs were non-recordable. The fundus appearance varied with pigmentary changes in the mid-periphery and atrophic

macular lesions. Some of the patients had cataracts and keratoconus.

13.6.3 LCA5 in Japanese/Asian Populations

No LCA5 patient has been reported in Japanese population. Fourteen Korean LCA patients who were found not to have mutations in known LCA causative genes also had no pathogenic mutation in the *LCA5* gene [99]. Results of comprehensive genetic investigations on Chinese patients with LCA/EORD indicated that the prevalence of LCA5 was 3% [17, 64, 114].

13.7 LCA6

LCA6 is caused by a homozygous mutation or compound heterozygous mutations of the retinitis pigmentosa GTPase regulator-interacting protein 1 (*RPGRIP1*) gene. LCA6 accounts for about 5% of all LCAs [24, 105, 118], and mutations in the *RPGRIP1* gene also cause autosomal recessive cone-rod dystrophy (CORD13).

13.7.1 Function of *RPGRIP1* Gene

The *RPGRIP1* gene is located on the long arm of chromosome 14 at locus 11. This gene is required for the normal morphogenesis and organization of the discs in the outer segment of the photoreceptors. The RPGRIP1 protein is present in the ciliary structure connecting the inner and outer segments of cones and rods, and it interacts with the retinitis pigmentosa GTPase regulator (*RPGR*) gene to promote outer segment development (Fig. 13.1) [120].

13.7.2 Clinical Features of LCA6

The phenotype of LCA6 is cone-rod dysfunction (Type 1 LCA). Generally, the fundus abnormality is relatively mild in patients with LCA6 compared to other types of LCA. The macular changes are minimal although retinal pigment mottling can be seen in the periphery [43, 48].

Dryja et al. [28] first reported on a 15-year-old patient with LCA6 who had poor visual acuity, hyperopia, nystagmus, and photophobia. The patient had pigmentary retinal degeneration in the periphery, but his 2-year-old affected sister had normally appearing fundus.

13.7.3 LCA6 in Japanese/Asian Populations

Suzuki et al. [104] reported on two Japanese brothers with LCA6. They had normal fundus for 10 years from the age of 2 years (Fig. 13.3). Their BCVAs were 0.06 OU and 2.4 cpd/55 cm OU measured with the Teller Acuity Cards. The older brother had high astigmatism and younger brother was hyperopic. The older brother reported day blindness but no night blindness. His flash ERGs were subnormal, and the flicker ERGs were non-recordable indicating a severe impairment of the cone system. Genetic analysis revealed a homozygous large deletion mutation of c.2710+372_2895+76del1339 in the exon 17 of the *RPGRIP1* gene, which was novel.

In Korea, one LCA6 patient was reported by Seong et al. [98].

Results of comprehensive genetic investigations on Chinese patients with LCA/EORD showed that the prevalence of LCA6 was 7% which is almost the same as the Caucasian population [17, 64, 114].

13.8 LCA7

LCA7 is caused by a heterozygous mutation in the cone-rod homeobox (*CRX*) gene, and this is with LCA11 and *OTX2*-associated LCA, the only three types of LCA that have a dominant inheritance pattern among the more than 20 types of LCA (Table 13.1). Prevalence of LCA7 is estimated to be 1–2% in all LCA/EORD. *CRX* is a relatively rare cause for LCA/EORD [24, 105, 118]. Mutations in this gene can also cause autosomal dominant cone-rod dystrophy (CORD2).

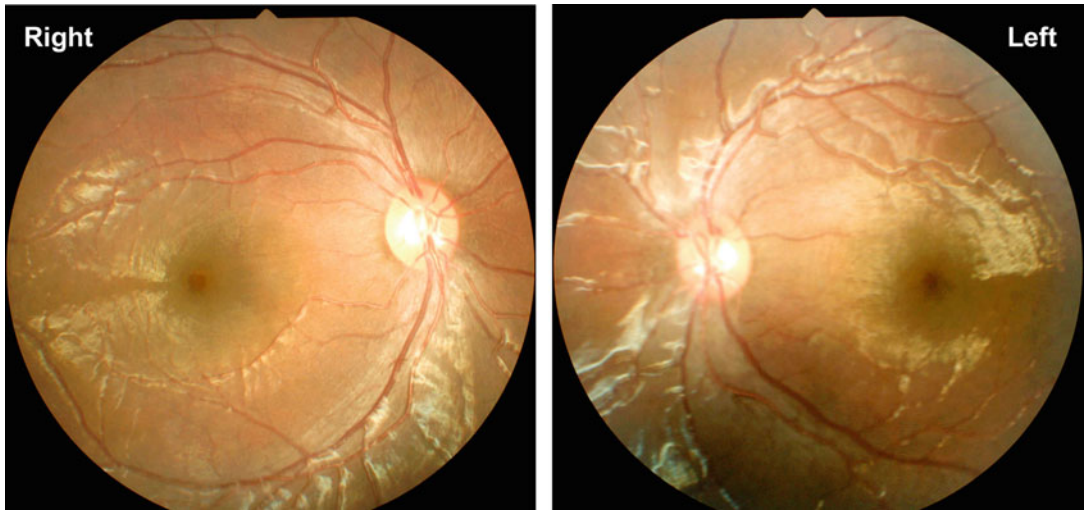

Fig. 13.3 Fundus photographs of a Japanese patient with the *RPGRIP1* mutations (LCA6)
This patient was reported by Suzuki et al. in 2014 [104]. The fundus photographs were taken at the age 2 years. The BCVA was 2.4 cpd/55 cm determined by the Teller Acuity Cards. These photographs were provided by Dr. Takuro Fujimaki and Dr. Akira Murakami and reproduced from report by Suzuki et al. [104] with permission

13.8.1 Function of *CRX* Gene

The *CRX* gene is located on the long arm of chromosome 19 at locus 13.

Furukawa et al. [34] discovered that the *Crx* gene is a photoreceptor-specific transcription factor which provides instructions for producing a protein that regulates the activity of other genes. The CRX protein belongs to the OTX family of homeodomain proteins including OTX1, OTX2, and OTX5 (CRX). CRX plays an important role in the differentiation of the photoreceptor cells especially the formation of the rod outer segments. CRX acts with other transcription factors such as, NRL, OTX2, and TR-β2.

13.8.2 Clinical Features of LCA7

The phenotype of patients with LCA7 is characterized by macular degeneration or macular atrophy, and the vision is usually poor [41]. The fundus of patients with LCA7 can be normal in infancy [27], and the fundus in the older patient is similar to that in patient with cone-rod dystrophy or retinitis pigmentosa [93]. Dharmaraj et al. [27] and Koenekoop et al. [53] reported that the vision was stable or even improved with increasing age in patients with LCA7.

Hull et al. [41] reported on the clinical findings of 18 patients with heterozygous mutations in the *CRX* gene. Their patients included four with LCA, five with cone-rod dystrophy, one with cone dystrophy, two with rod-cone dystrophy, and six with nonspecific macular atrophy.

13.8.3 LCA7 in Japanese/Asian Populations

Nakamura et al. [75] reported on a 16-year-old Japanese boy with LCA7 who had a heterozygous de novo mutation in the *CRX* gene. The mutation was a frameshift mutation of A174 [1-bp del] which was novel. The vision of the patient was less than 0.02 since infancy. His fundi had coloboma-like macular atrophy with attenuated retinal vessels and diffuse retinal mottling (Fig. 13.4). The ERGs were non-recordable.

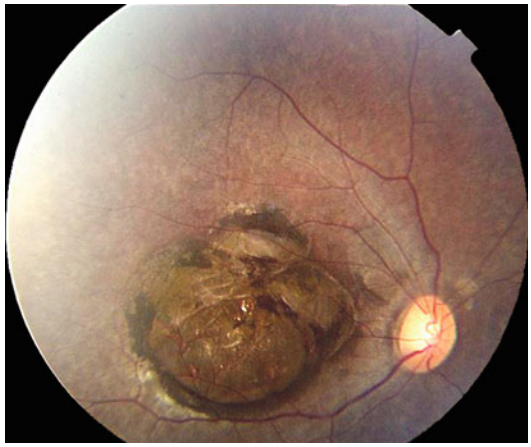

Fig. 13.4 Fundus photographs of a Japanese patient with the *CRX* de novo mutation (LCA7)
This patient was reported by Nakamura et al. in 2002 [75]. He was a 16-year-old boy with BCVA of less than 0.02. This photograph was provided by Dr. Makoto Nakamura and reproduced from report by Nakamura [76] with permission

Recently two Chinese patients with LCA7 were reported [121]. The authors examined 109 probands with LCA/EORD genetically and found de novo *CRX* mutations in two patients. Results of comprehensive genetic investigations of Chinese patients with LCA/EORD showed the prevalence of LCA7 was 2–3% [17, 64, 114].

13.9 LCA8

LCA8 is caused by a homozygous mutation or compound heterozygous mutations in the crumbs homolog 1 (*CRB1*) gene. Cases of LCA8 account for 8% of the LCA cases [24, 105, 118], and they have various clinical phenotypes, e.g., autosomal recessive RP (RP12), autosomal recessive retinal telangiectasia with exudation (Coats-like vasculopathy; [23]), and autosomal dominant pigmented paravenous chorioretinal atrophy (PPCRA).

13.9.1 Function of *CRB1* Gene

The *CRB1* gene is located on the long arm of chromosome 1 at locus 31.3.

The CRB1 transmembrane protein is located at a subapical region above the intercellular junctions between the photoreceptors and Müller cells. This gene is important for maintaining the polarity of the photoreceptor cells during retinal development [81, 109].

13.9.2 Clinical Features of LCA8

The phenotype of *CRB1*-associated retinopathy varies from typical LCA, RP12, Coats-like vasculopathy, to PPCRA.

Lotery et al. [67] reported on the clinical features of 19 patients with LCA8. Eighteen patients had nystagmus, and their vision ranged from light perception to 0.4, and 16 patients had vision 0.1 or worse. Most patients had hyperopia greater than +5.0 diopters. Ophthalmoscopy showed diffuse retinal degeneration. Coloboma-like macular atrophy was found in three patients. Periarteriolar preservation of the RPE was detected in two siblings, and keratoconus was present in a different pair of siblings.

Jacobson et al. [42] reported that OCT images in patients with LCA8 showed thickened and coarsely laminated retinas which are unique findings among LCAs. They suggested that the coarsely laminated retinas resulted from perturbed signal communications between the photoreceptors and Müller cells during retinal laminar development.

The visual impairments in patients with LCA8 progress slowly and most of the vision is reduced to counting finger or worse in the fourth decade of life [38].

13.9.3 LCA8 in Japanese/Asian Populations

Kuniyoshi et al. [58] reported on two unrelated Japanese boys with LCA8. They were followed clinically for 7 and 11 years. Initially their fundi appeared as whitish marbled degeneration of the RPE; however the attenuation of the retinal vessels was not severe (Fig. 13.5). With increasing time, pigmentations appeared in the whitish

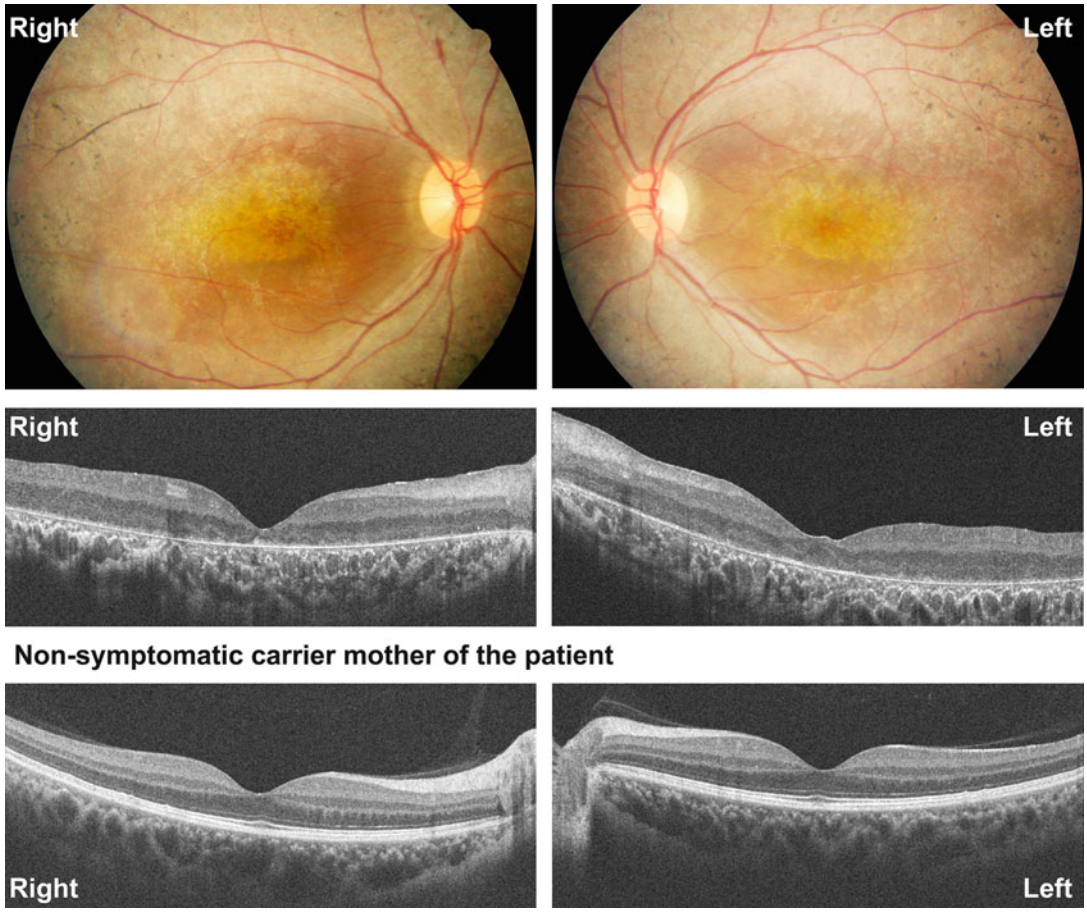

Fig. 13.5 Fundus photographs and optical coherence tomographic (OCT) images of a Japanese patient with the *CRB1* mutations (LCA8) and his non-symptomatic carrier mother
This patient was reported by Kuniyoshi et al. in 2015 [58]. The fundus photographs (*upper row*) were taken when he was 9 years old. His decimal BCVA was 0.3 in both eyes. OCT images showed thickened and coarsely laminated retina (*middle row*), whereas non-symptomatic carrier mother showed normal OCT findings (*lower row*). These photographs are reproduced from our report [58] with permission

lesions, and visual field tests detected a ring scotoma in both patients. OCT images showed a thickened and disorganized retina which is the typical findings of eyes with LCA8 (Fig. 13.5). Next-generation sequence on whole exome revealed p.R632X nonsense and c.652+1_652+4delGTAA splice site mutations in a patient and c.652+1_652+4delGTAA and c.652+1_652+2insT splice site mutations in another patient, in the *CRB1* gene.

In Korea, one LCA8 patient was reported by Seong et al. [98].

Results of the comprehensive gene investigations on Chinese patients with LCA/EORD revealed that the prevalence of LCA8 was 13% [17, 64, 114]. This indicates the *CRB1* gene is one of the most common causative genes for LCA/EORD in the Chinese population, differently from the Caucasian population.

13.10 LCA9

LCA9 is caused by a homozygous mutation or compound heterozygous mutations in the nicotinamide nucleotide adenylyltransferase 1 (*NMNAT1*) gene. The prevalence of mutations

in the *NMNAT1* in patients with LCA is unknown because this gene was only recently found to be causative for LCA [55, 87].

13.10.1 Function of *NMNAT1* Gene

This gene is located on the short arm of chromosome 1 at locus 36.22. The NMNAT1 protein is an enzyme involved in nicotinamide adenine dinucleotide (NAD) biosynthesis, and it catalyzes the formation of NAD^+ from nicotinamide mononucleotide and ATP in the nucleus [3]. It is a stress-response protein and acts as a chaperone for neural maintenance and protection [6].

13.10.2 Clinical Features of LCA9

The typical phenotype of LCA9 is severe and early-onset vision reduction with macular degeneration and atrophy [55, 87]. The peripheral retina is also degenerated to different degrees.

Koenekoop et al. [55] reported on 14 patients with mutations in *NMNAT1*, and all of them had severe LCA but were physically and mentally healthy. In addition, all had a coloboma-like macular atrophy. Perrault et al. [87] also reported on the early and rapid formation of the coloboma-like macular atrophy in patients LCA9.

13.10.3 LCA9 in Japanese/Asian Populations

Coppieters et al. [20] reported four families associated with LCA9, and two of them were Japanese families. Both of the probands in these two unrelated families were 3-month-old boys. They had nystagmus and night blindness but photophobia was not present (Type 2 LCA). They had circular degeneration of the macula that is commonly seen in patients with LCA9.

Genetic study in large cohort of 243 unrelated Chinese patients with LCA/EORD revealed only three patients had pathogenic mutations in the *NMNAT1* gene [17, 64, 114]. The *NMNAT1* gene seems to be rare cause of LCA/EORD in the Asian population as it in the European/American populations.

13.11 LCA10

LCA10 is caused by a homozygous mutation or compound heterozygous mutations in the centrosomal protein 290 kDa (*CEP290*) gene, also called the nephronophthisis 6 (*NPHP6*) gene. LCA10 is one of the most common types of LCA accounting for 6–22% of all patients with LCA [24, 86, 105, 118]. This gene is also causative for a broad spectrum of diseases, e.g., the Joubert syndrome 5 (JBTS5), Senior-Løken syndrome 6 (SLSN6), and Meckel syndrome 4 (MKS4).

13.11.1 Function of *CEP290*

This gene is located on the long arm of chromosome 12 at locus 21.32. *CEP290* encodes a centrosomal and ciliary protein which is expressed ubiquitously in ciliated cells including cones and rods (Fig. 13.1). The centrosomes are an assembly of microtubules which transport materials in cells and maintain the cell shape. CEP290 interacts with several microtubule-based transport proteins including RPGR (mutated in patients with X-linked RP and cone-rod dystrophy), RPGRIP1 (LCA), CC2D2A (JBTS and MKS), and IQCB1 (SLSN) [16].

13.11.2 Clinical Features of LCA10

The typical phenotype of LCA10 is cone-rod dysfunction (Type 1 LCA) [86]. Perrault et al. [86] reported their clinical findings in 47 patients of 40 families associated with LCA10. Systemically, hypotonia or ataxia was noted in four patients, and mental retardation or autism was noted in six families. All patients had severe cone-rod dysfunction, and all had salt-and-pepper retina with macular degeneration. Most of the patients had visual acuity poorer than 0.05, and

they progressed to typical RP in the first decade of life. They had high hyperopia and slight photophobia at the end of the second decade of life.

13.11.3 LCA10 in Japanese/Asian Population

No patient with LCA10 has been reported from Japan although LCA10 is the most common type of LCA in the Caucasian population.

Interestingly, results of comprehensive gene investigation on Chinese patients with LCA/EORD showed prevalence of LCA10 was 7%, which is considerably lower than that in the European/American populations [17, 64, 114].

13.12 LCA11

LCA11 is caused by a heterozygous mutation of the inosine 5′-monophosphate dehydrogenase 1 (*IMPDH1*) gene, and it has an autosomal dominant inheritance pattern. Although the prevalence of this mutation among the patients with LCA has not been reported, *IMPDH1* appears to be a rare cause of LCA. This gene is known to be the cause of autosomal dominant RP (RP10).

13.12.1 Function of *IMPDH1* Gene

This gene is located on the long arm of chromosome 7 between loci 31.3 and 32. *IMPDH* is a housekeeping gene which is ubiquitously expressed in all eukaryotes and most prokaryotes. The IMPDH protein is an enzyme which catalyzes the rate-limiting step in the de novo synthesis of guanine nucleotide. Guanine plays an important role in the visual cycle. Guanine is converted to GMP and the concentration of the cGMP in the outer segments of the photoreceptors is controlled for responding to photostimulation (Fig. 13.1) [12, 19, 111].

13.12.2 Clinical Features of LCA11

Bowne et al. [12] reported on two unrelated infants. The first patient was an 8-month-old boy, who was diagnosed with LCA and had delayed development and severe hypotonia. He had no visual fixation and had nystagmus and digito-ocular signs. His fundi had diffuse RPE mottling with no pigmentary deposits. A macular reflex was present in both eyes. The second patient was a 33-month-old infant girl who was noticed not to see things in her peripheral visual field, and she could not find her food in dim lighting. Her visual acuity was 0.5 with hyperopic refractive errors. Her fundi were depigmented with vascular attenuation and disc pallor.

13.12.3 LCA11 in Japanese/Asian Populations

LCA11 has not been reported in the Japan population although juvenile-onset RP associated with an *IMPDH1* mutation was reported by Wada et al. [112]. They reported on two unrelated families with four severely affected individuals. The inheritance pattern was autosomal dominant. The RP progressed to legal blindness after 10 years of age, and the individuals had severe diffuse retinal degeneration with atrophic maculae.

Chen et al. [17] reported on a Chinese patient with LCA/EORD with *IMPDH1* mutations.

13.13 LCA12

LCA12 is caused by a homozygous mutation of the retinal degeneration 3 (*RD3*) gene. The prevalence of this mutation among LCA patients is unknown, but it appears to be very low because Perrault et al. [89] reported that only 11 patients were found to have *RD3* mutations in 852 patients with LCA/EORD or autosomal recessive RP.

13.13.1 Function of *RD3* Gene

This gene is located on the long arm of chromosome 1 at locus 32.3. The *RD3* gene encodes a retinal protein highly expressed in the outer segments of the photoreceptors where guanylate cyclase 1 (GC1, GUCY2D) and GC2 (GUCY2F) are localized in cones and rods, respectively (Fig. 13.1). Azadi et al. [7] concluded that RD3 may be an accessory protein required for trafficking the vesicle of GC1 and GC2 from the inner to the outer segments of the photoreceptors, and it may modulate the enzymatic activity of GC.

13.13.2 Clinical Features of LCA12

The phenotype of LCA12 is typical of LCA1, namely, poor vision, congenital nystagmus, sluggish pupillary reflexes, digito-ocular signs, photoaversion, and hyperopia [89]. Preising et al. [91] reported his findings on four siblings with LCA12 from a large consanguineous Kurdish family. All of them had congenital nystagmus, glare sensitivity, and vision worse than 0.08 with hyperopic refractive errors. The fundus was unremarkable before age of 2 years; however retinal degeneration with attenuated vessels, RPE mottling, and macular degeneration were noted at ages older than 2 years.

13.13.3 LCA12 in Japanese/Asian Populations

No patient has been reported in Japan. Genetic study in large cohort of 243 unrelated Chinese patients with LCA/EORD revealed only two patients had pathogenic mutations in the *RD3* gene [17, 64, 114]. The *RD3* gene seems to be rare cause of LCA/EORD in the Asian population as it in the European/American populations.

13.14 LCA13

LCA13 is caused by a homozygous mutation or combined heterozygous mutations in the retinol dehydrogenase 12 (*RDH12*) gene. LCA13 accounts for 4% of all LCAs [24, 105, 118]. This gene is also causative for autosomal dominant/recessive RP (RP53).

13.14.1 Function of *RDH12* Gene

This gene is located on the long arm of chromosome 14 at locus 24.1. RDH has several isoforms which are expressed in the RPE and retina. Each subtype of RDH has oxidoreductive catalytic activities in the retinoid cycle. RDH12 is a key enzyme for converting all-*trans* retinal to all-*trans* retinol (vitamin A) in the retinoid cycle (Fig. 13.1). RDH12 might be the key enzyme in the formation of 11-*cis* retinal from 11-*cis* retinol during the regeneration of the cone visual pigments [46, 95].

13.14.2 Clinical Features of LCA13

The typical clinical picture of *RDH12*-associated retinopathy is a rod-cone dystrophy with diffuse retinal pigment clumping and atrophic yellow macula (Type 2 LCA).

Perrault et al. [84] reported on 12 patients in 8 families with LCA13. All of the patients had a transient improvement of the visual acuity in the early stages of the disease as it did with the LCA2 (*RPE65*). They had a progressive rod-cone dystrophy with severe macular atrophy.

Mackay et al. [69] reported on the clinical features of *RDH12* retinopathy in 32 patients with the *RDH12* mutation. A severe visual reduction was noted at birth in 5 patients; however the other 27 patients did not have a decline of vision until ages 1–5 years.

13.14.3 LCA13 in Japanese/Asian Populations

Kuniyoshi et al. [57] reported on the longitudinal clinical course of three Japanese patients with LCA13: two siblings in one family and a girl in another unrelated family. All of them had a

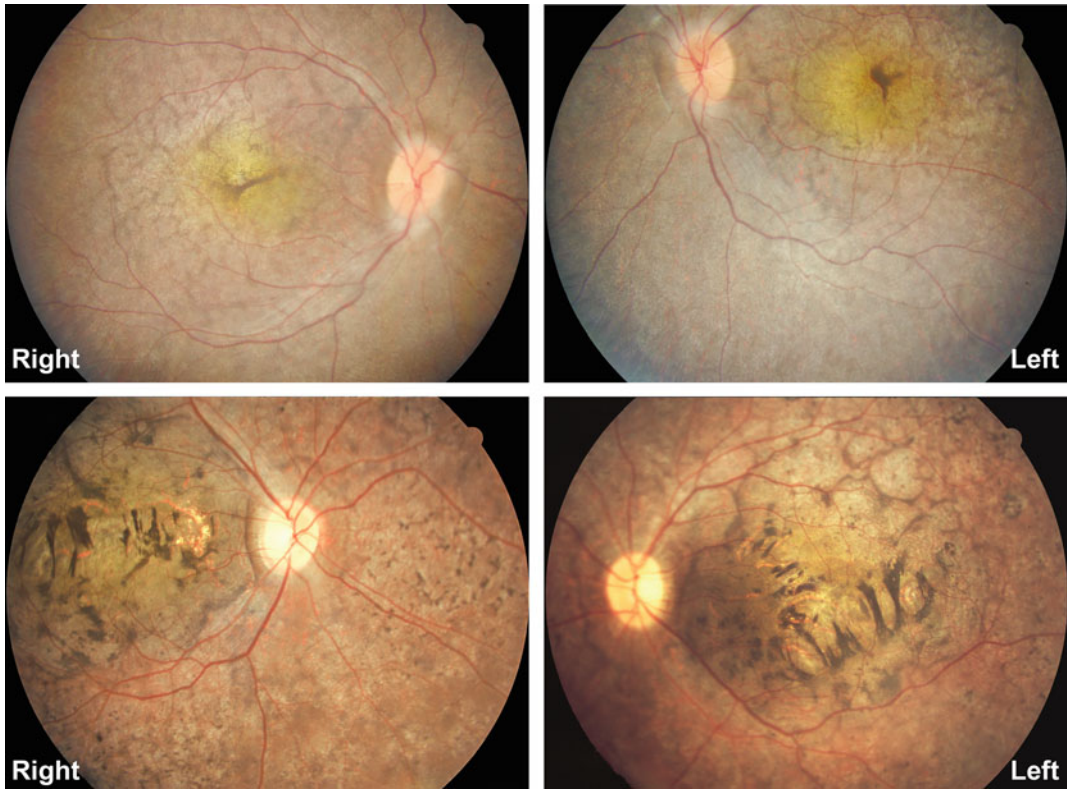

Fig. 13.6 Fundus photographs of Japanese patients with the *RDH12* mutations (LCA13)
These two unrelated children were reported by Kuniyoshi et al. in 2014 [57]: a 4-year-old girl (*upper row*) and 8-year-old boy (*lower row*). The decimal BCVA of the girl was 0.07 in both eyes and that of the boy was 0.09 in the right eye and 0.3 in the left eye. These photographs are reproduced from our report [57] with permission

homozygous p.A126V substitution in the *RDH12* gene. They were clinically observed for 10–28 years, and their vision decreased from 0.06 or 1.0 to counting finger or light perception at age 8 years or high teens. Their fundi were typical of *RDH12*-associated retinopathy, namely, mottled RPE, clumped pigmentation, and yellow and atrophic maculae (Fig. 13.6).

Results of comprehensive genetic investigations on Chinese patients with LCA/EORD showed that the prevalence of LCA13 was 3%, which was almost the same as in the Caucasian population [17, 64, 114].

13.15 LCA14

LCA14 is caused by a homozygous mutation in the lecithin retinol acyltransferase (*LRAT*) gene. The prevalence of LCA14 is estimated to be about 1% among all patients with LCA [24, 105, 118]. This gene is also known to cause autosomal recessive EORD/juvenile RP.

13.15.1 Function of *LRAT* Gene

This gene is located on the long arm of chromosome 4 at locus 32.1 and is expressed in the RPE and liver. This enzyme catalyzes the change of all-*trans* retinol (vitamin A) to all-*trans* retinyl esters in the RPE as part of the retinoid cycle (Fig. 13.1). This is followed by the synthesis of 11-*cis* retinol by RPE65 and 11-*cis* retinal by RDH5 which results in the regeneration of the chromophores for rhodopsin and the cone photopigments (Fig. 13.1) [46, 95].

13.15.2 Clinical Features of LCA14

The typical phenotype of LCA14 is a rod-cone dystrophy with peripheral retinal degeneration and constricted visual fields (Type 2 LCA) [108]. These findings are similar to those in *RPE65* retinopathy, and RPE65 also plays an important role in the visual cycle.

Dev Borman et al. [25] reported detailed phenotypes of four patients with *LRAT*-associated EORD whose ages ranged from 27 to 54 years at the time of the examinations. Their visual symptom began at the age of 1–3 years, and three of them complained of severe night blindness. None of them was noted to have nystagmus in the first year of life. Their BCVAs ranged from hand motion to 0.6. Fundus examinations revealed RPE atrophy throughout the retina with arteriolar attenuation; however the pigmentation in the retina was minimal. The macular region was relatively intact in two of the patients. The rod ERGs were non-recordable, and small cone ERGs were recorded in two patients.

13.15.3 LCA14 in Japanese/Asian Populations

LCA14 has not been reported in the Asia population including Japan, Korea, and China, except one patient in the report by Dev Borman et al. [25] was from Southeast Asia.

13.16 LCA15

LCA15 is caused by a homozygous mutation or compound heterozygous mutations in the tubby-like protein 1 (*TULP1*) gene. LCA15 makes up 1% of all patients with LCA [24, 105, 118]. This gene is also causative for autosomal recessive RP (RP14) and cone dysfunction [94].

13.16.1 Function of *TULP1* Gene

This gene is located on the short arm of chromosome 6 at locus 21.31. *TULP1* encodes a protein which belongs to the tubby protein family, tubby, TULP1, 2, and 3. Tubby and TULP3 are expressed widely in the central nervous system, but TULP1 is restricted to the retina and TULP2 to the testis. TULP1 is a cytoplasmic protein and is expressed in the photoreceptor cells especially in the inner segments, the connecting cilium, perinuclear cytoplasm, and synaptic terminals. TULP1 enhances the phagocytosis by the RPE which removes cellular debris and apoptotic cells (Fig. 13.1) [14, 15]. TULP1 is also involved in the vesicular trafficking of photoreceptor proteins. TULP1 may be required for normal synaptic function [119].

13.16.2 Clinical Features of LCA15

Generally, a *TULP1* mutation causes predominantly rod-mediated diseases, and therefore patients with LCA15 complain of night blindness and constricted visual fields (Type 2 LCA).

Jacobson et al. [44] reported detailed phenotypes of five patients with LCA15. Their age ranged 4–36 years, and their BCVA ranged from hand motion to 0.2. All of them had a history of childhood-onset night vision and visual field disturbances. Nystagmus was present to varying degrees in all but one patient. The appearance of the peripheral retina ranged from no pigment migration to dense pigmentary retinopathy.

13.16.3 LCA15 in Japanese/Asian Populations

No LCA15 patient has been reported in Japan. In Asian population, Chen et al. [17] reported on one Chinese patient and Wang et al. [114] reported on four Chinese patients with LCA15. The prevalence of LCA15 in the Chinese population is estimated to be 2% [17, 64, 114].

13.17 LCA16

LCA16 is caused by a homozygous mutation in the potassium channel, inwardly rectifying subfamily J, member 13 (*KCNJ13*) gene. The prevalence of mutations in this gene among the

patients with LCA has not been reported. This gene is also causative for snowflake vitreoretinal degeneration (SVD; [39]). SVD has an autosomal dominant inheritance pattern which is different from that of LCA16.

13.17.1 Function of *KCNJ13* Gene

The *KCNJ13* gene is located on the long arm of chromosome 2 at locus 37. This gene encodes a protein in the RPE, Kir7.1, which is a member of the inwardly rectifying potassium (Kir) channel family. Kir7.1 channels are located in the RPE apical process membrane which surrounds the outer segments of the photoreceptors (Fig. 13.1). Kir7.1 plays a key role in the transport of ions, metabolites, and fluid between the subretinal space and the RPE cells. Kir7.1 forms ion channel pores which return potassium to the subretinal space, together with the sodium-potassium adenosine triphosphatase pump (Na^+/K^+-ATPase or Na^+/K^+ pump) which allows potassium ions to pass into the RPE cell [59].

13.17.2 Clinical Features of LCA16

Only two reports have been published on patients with LCA16, and both were studies on patients of Middle Eastern origin [80, 100]. The patient reported by Pattnaik et al. [80] was an 8-year-old boy who was noted to have difficulty with night vision by the age of 2 years and had poor central vision by school age. His BCVAs at age 8 years were 0.1 in the right eye and 0.05 in the left eye. Fundus examinations at age 8 showed RPE mottling in the macula with arteriolar attenuation. His vision was reduced at 10 year of age when macular pigmentation appeared.

13.17.3 LCA16 in Japanese/Asian Populations

No patient with LCA16 has been reported in the Asian population including the Japanese.

13.18 LCA17

LCA17 is caused by compound heterozygous mutations in the growth differentiation factor 6 (*GDF6*) gene. The prevalence of this mutation among patients with LCA is not known because this gene was only recently found to be causative for LCA [5]. This gene is also causative for Klippel-Feil syndrome 1 (KFS1), isolated microphthalmia with coloboma 6 (MCOPCB6), and isolated microphthalmia 4 (MCOP4).

13.18.1 Function of *GDF6* Gene

The *GDF6* gene is located on the long arm of chromosome 8 at locus 22. The growth differentiation factors (GDFs) and bone morphogenetic proteins (BMPs) are part of the transforming growth factor beta (TGFβ) superfamily. These proteins are essential for axis formation, cell fate determination, and patterning of neurons, muscles, bones, and cartilages. In the eye, GDF6 is involved in the development of the eyes including the retina and most likely plays a role in the survival of the photoreceptor cells [5, 79]

13.18.2 Clinical Features of LCA17

Only one paper has been published on LCA17 because most of the *GDF6* mutations cause syndromic or non-syndromic microphthalmia with/without coloboma. Asai-Coakwell et al. [5] found 1 patient with LCA17 after screening DNA samples of 179 patients with LCA/EORD who were previously screened for known LCA causative genes. The BCVA of this patient was hand motions, and the ERGs were non-recordable. No other ocular or systemic phenotype was noted. Her parents had reduced and delayed rod b-waves but had normal fundi. The fundus findings of the patient were not presented.

13.18.3 LCA17 in Japanese/Asian Populations

No patient with LCA16 has been reported in the Asian population including the Japanese.

13.19 LCA18

LCA18 is caused by a homozygous mutation in the peripherin 2 (*PRPH2*) gene which was formerly called retinal degeneration slow (*RDS*) or peripherin/*RDS* gene. On the other hand, heterozygous mutations in this gene are causative for RP (RP7), retinitis punctata albescens (albipunctate retinal dystrophy), and various types of macular dystrophy, including vitelliform macular dystrophy (VMD3), central areolar choroidal dystrophy (CACD2), and patterned macular dystrophy (MDPT1).

13.19.1 Function of *PRPH2* Gene

The *PRPH2* gene is located on the short arm of chromosome 6 at locus 21.1 and encodes a membrane glycoprotein, peripherin 2. Peripherin 2 is present in the disk rims, the hairpin membrane of the discs, of the outer segments of both cones and rods (Fig. 13.1). It is also involved in the formation, stability, and compacting of the structures of the outer segments together with retinal outer segment membrane protein 1 (ROM1) [4, 66].

13.19.2 Clinical Features of LCA18

Wang et al. [115] screened 179 unrelated patients with LCA or juvenile RP and found homozygous missense mutations of the *PRPH2* in 3 patients. All had visual impairments that began within the first year of life, two had nystagmus, the ERGs were reduced or non-recordable, and maculopathies with peripheral retinal degeneration were present. Their family members who carried heterozygous mutations in the *PRPH2* gene were asymptomatic but had maculopathy. This was the first and only the report on LCA18.

13.19.3 LCA18 in Japanese/Asian Populations

No patient has been reported with LCA18 in the Asian populations including the Japanese, although the findings in several Japanese patients with cone-rod dystrophy or macular degeneration have been published (Nakazawa et al. [77, 78]).

13.20 LCA with *OTX2* Gene Mutation

LCA/EORD with or without pituitary dysfunction can be caused by heterozygous mutations in the orthodenticle homeobox 2 (*OTX2*) gene, and it has an autosomal dominant inheritance pattern. The prevalence of LCA caused by mutations of the *OTX2* gene among patients with LCA has not been determined because only a few studies have been published on *OTX2*-associated LCA [37, 92, 114]. This gene is also causative for syndromic microphthalmia 5 (MCOPS5) and combined pituitary hormone deficiency 6 (CPHD6) and dominant pattern dystrophy.

13.20.1 Function of *OTX2* Gene

This gene is located on the long arm of chromosome 14 at locus 22.3. Mammals have three kinds of *Otx* genes, namely, *Otx1*, *Otx2*, and *Otx5* (*Crx*), all of which synthesize transcription factors. The *OTX* genes are part of a family of homeobox genes which act during early embryonic development and control the formation of many body structures. The *OTX* genes play a critical role in the development of the eyes, optic nerves, and brain including the pituitary gland [56, 79].

13.20.2 Clinical Features of *OTX2*-Associated LCA/EORD

The common phenotype caused by an *OTX2* mutation is anophthalmia or microphthalmia with a delay of ocular development.

Henderson et al. [37] screened 142 patients with LCA/EORD and found a 7-year-old boy with a heterozygous de novo nonsense mutation in the *OTX* gene. His parents reported that the boy had poor vision and nyctalopia during his first year of life. He had mild torsional

nystagmus, and his decimal BCVA at the age of 6 years was 0.3–0.4, and color vision was normal. Ophthalmoscopy showed fine granular pigmentation of the RPE throughout the fundus, but vascular attenuation was not present. The rod ERGs were non-recordable, the cone ERGs were reduced and delayed, and the bright flash ERGs were the negative type.

13.20.3 LCA Associated with *OTX2* Mutation in Japanese/Asian Populations

No *OTX2*-associated LCA/EORD has not been reported in Eastern Asia, except one Chinese patient reported by Wang et al. in 2015.

13.21 LCA with *IQCB1* Gene Mutation

Homozygous or compound heterozygous mutations of the IQ motif-containing protein B1 (*IQCB1*) gene, also called nephronophthisis 5 (*NPHP5*) gene, can cause LCA/EORD. The prevalence of LCA with *IQCB1* among patients with LCA has not been determined. This gene was previously known to be causative for Senior-Løken syndrome 5 (SLSN5) which is characterized by nephronophthisis and tapetoretinal degeneration.

13.21.1 Function of *IQCB1* Gene

This gene is located on the long arm of chromosome 3 at locus 13.33, and it encodes a nephrocystin protein that interacts with calmodulin and the retinitis pigmentosa GTPase regulator (RPGR) protein. IQCB1 is localized to the primary cilium of the renal epithelial cells and connecting cilium of the photoreceptor cells (Fig. 13.1). Together with CEP290 (NPHP6), they regulate the transport of molecules from the inner to the outer segments of the photoreceptors [16, 96].

13.21.2 Clinical Features of *IQCB1*-Associated LCA

Most patients with *IQCB1*-associated retinopathy have severe renal dysfunction.

Stone et al. [103] and Estrada-Cuzcano et al. [30] reported homozygous or compound heterozygous mutations of the *IQCB1* gene in patients with LCA/EORD. The phenotype of the patients resembled that of patients with non-syndromic classical LCA with early-onset pendular nystagmus, poor fixation at birth, and non-recordable rod and cone ERGs at the early stage of the disease. The patients were hyperopic and their decimal BCVAs ranged from 0.25 to light perception at the age of 1–23 years.

13.21.3 LCA Associated with *IQCB1* in Japanese/Asian Populations

No patient with *IQCB1*-associated LCA has been reported in Japan. Chen et al. [17] and Wang et al. [114] reported 1 and 2 patients with *IQCB1*-associated LCA among 243 unrelated Chinese patients with LCA/EORD.

13.22 LCA with *IFT140* Gene Mutation

Khan et al. [50] reported an LCA patient with homozygous mutation of the intraflagellar transport 140 (*IFT140*) gene. Mutations of this gene were known to cause short-rib thoracic dysplasia 9 with or without polydactyly (*SRTD9*).

13.22.1 Function of *IFT140* Gene

This gene is located on the short arm of chromosome 16 at locus 13.3, and it encodes a subunit of the intraflagellar transport complex A (IFTA) which is involved in retrograde ciliary transport in cells of the kidneys, liver, brain, and photoreceptors [18, 88]. IFT plays an important role in the formation and maintenance of the

connecting cilia between the inner and outer segments of the photoreceptors. The cilia also play a role in skeletal and chondral development, although the mechanism is not well understood [97].

13.22.2 Clinical Features of *IFT140*-Associated LCA/EORD

The phenotype associated with an *IFT140* mutation is "ciliopathy" [10], and therefore most of these patients have syndromic disorders including skeletal abnormalities with or without ocular manifestations.

Khan et al. [50] reported two unrelated boys with *IFT140*-associated LCA. They were noted to have poor vision and nystagmus soon after birth. Their fundi had peripheral RPE mottling with multiple depigmented spots in both eyes. The ERGs were non-recordable. Systemic X-rays showed cone-shaped epiphyses of the hands and relatively short bone lengths.

13.22.3 LCA Associated with *IFT140* Mutation in Japanese/Asian Populations

No patient has not been reported with *IFT140*-associated retinopathy in the Asian population including the Japanese.

13.23 LCA with *PNPLA6* Gene Mutation

Kmoch et al. [52] reported that compound heterozygous mutations in the patatin-like phospholipase domain-containing protein 6 (*PNPLA6*) gene, previously referred to as the neuropathy target esterase (*NTE*) gene, can cause LCA. This gene was known to be causative of Oliver-McFarlane syndrome (OMCS), Boucher-Neuhauser syndrome (BNHS), spastic paraplegia 39 (SPG39), and Laurence-Moon syndrome (LNMS).

13.23.1 Function of *PNPLA6* Gene

This gene is located on the short arm of chromosome 19 at locus 13.2. The *PNPLA6* gene encodes a phospholipase that deacetylates intracellular phosphatidylcholine (PC) to lysophosphatidylcholine (LPC) and LPC to glycerophosphocholine in the central nervous system. It is believed to be involved in the regulation of membrane lipid homeostasis and in the survival of both neurons and glia [74].

13.23.2 Clinical Features of *PNPLA6*-Associated LCA/EORD

Kmoch et al. [52] reported on a Caucasian patient with LCA caused by compound heterozygous mutations in the *PNPLA6* gene. He had a congenital reduction of vision with nystagmus. He had no systemic symptoms except autism. His BCVA at age 20 was counting fingers, and the fundi were severely degenerated.

13.23.3 LCA/EORD Associated with *PNPLA6* Mutation in Japanese/Asian Populations

No patient has been reported with *PNPLA6*-associated retinopathy in the Asian population including the Japanese.

13.24 LCA with *PEX1* Gene Mutation

Michelakakis et al. [71] reported that homozygous mutations in the peroxisomal biogenesis factor 1 (*PEX1*) gene can cause an LCA phenotype. This gene is also causative for peroxisome biogenesis disorder 1A (Zellweger) and 1B (NALD/IRD).

13.24.1 Function of *PEX1* Gene

This gene is located on the long arm of chromosome 7 at locus 21.2. The PEX1 protein is a type

of peroxins which are enzymes that are expressed in a cell organelle, peroxisome. The peroxins are needed to breakdown many different molecules including fatty acids and toxic compounds. They are also involved in the production of lipids used in the intestines and in the nervous system. Peroxins assist in the biogenesis of peroxisomes by producing membrane peroxisomes [79].

13.24.2 Clinical Features of *PEX1*-Associated LCA/EORD

Michelakakis et al. [71] reported on a 5.5-year-old girl who had the LCA phenotype. She was noted to fail to make visual contact with both parents and surroundings. Nystagmus was present. Ophthalmologic examinations revealed "salt-and-pepper" tapetoretinal degeneration with bilateral papilledema. She had mild facial dysmorphism, and neurologic examinations revealed a mild psychomotor retardation with cerebellar tract involvement. Genetic investigation concluded that her phenotype was caused by PEX1 deficiency.

13.24.3 LCA Associated with the *PEX1* Mutation in Japanese/Asian Populations

No patient has been reported with *PEX1*-associated retinopathy in the Asian population including the Japanese.

13.25 LCA with *CABP4* Gene Mutation

Aldahmesh et al. [2] reported that homozygous mutations in the calcium-binding protein 4 (*CABP4*) gene can cause LCA/EORD. This gene was previously known to be causative for autosomal recessive congenital stationary night blindness (CSNB2B) also called congenital nonprogressive cone-rod synaptic disorder (CRSD).

13.25.1 Function of *CABP4* Gene

This gene is located on the long arm of chromosome 11 at locus 13.2. The CABP family is a subfamily of calmodulin-like neuronal Ca^{++}-binding proteins. CABP4 is located at the synaptic terminals of photoreceptors and is involved in normal synaptic function by regulating the calcium influx and neurotransmitter release (Fig. 13.1) [35].

13.25.2 Clinical Features of *CABP4*-Associated LCA/EORD

Aldahmesh et al. [2] and Khan et al. [48, 49] reported the clinical characteristics of *CABP4*-related retinal dystrophy. All patients had congenital nystagmus, nonprogressive low vision, photophobia, and normal or near-normal fundus appearance. None had night blindness. The scotopic bright flash ERGs were the negative type, the rod responses were normal, but the cone responses were abnormal.

13.25.3 LCA Associated with *CABP4* Mutation in Japanese/Asian Populations

This unique juvenile retinal dystrophy has not been reported in the Eastern Asian population including the Japanese.

13.26 LCA with *MERTK* Mutation

Mackay et al. [68] reported that homozygous mutations of the MER tyrosine kinase proto-oncogene (*MERTK*) gene can cause childhood-onset rod-cone dystrophy. This gene is also known to be causative for autosomal recessive RP (RP38).

13.26.1 Function of *MERTK* Gene

MERTK is located on the long arm of chromosome 2 at locus 13. MER tyrosine kinase is a member of MER/AXL/TYRO3 receptor tyrosine kinase family, and it is expressed in the RPE and is required for phagocytosis (Fig. 13.1) [31].

13.26.2 Clinical Features of *MERTK*-Associated LCA/EORD

Mackay et al. [68] reported on two siblings who had rod-cone dystrophy. The elder brother was first noticed to have difficulties with night vision and was found to have visual field defects at 9 year of age. The younger brother was noticed to have difficulty seeing in the dark from an early age. Their BCVA was 0.02 and 0.5 at age of 26 years and 8 years, respectively. Fundus examinations showed peripheral retinal degeneration with macular atrophy. Genetic studies showed a homozygous deletion of exon 8 of the *MERTK* gene.

13.26.3 LCA Associated with *MERTK* Mutation in Japanese/Asian Populations

No patient with *MERTK*-associated retinopathy has been reported in the Japanese population. From China, Li et al. [64] reported two unrelated LCA patients were caused by *MERTK* mutations.

13.27 Summary

The genotypes and phenotypes in patients with LCA/EORD are summarized and correlated. Approximately 25 genes have been reported as causative for LCA/EORD, while 8 genes have been reported in the Japanese patients and 6 of them were reported in 2014 and 2015. Genetic examinations of Japanese patients with LCA/EORD revealed novel pathogenic mutations in several known genes; however the genetic bases for more than one-half of the Japanese patients with LCA have not been determined.

Recently, reports on genetic studies on large cohort of Chinese patients with LCA/EORD were published. The investigators discovered a "distinct" mutation spectrum in the Chinese population compared to those in the European/American populations.

New causative genes for LCA/EORD, some of them may be specific in the Japanese/Asian populations, should be discovered in the near future.

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Paradigm of Susceptibility Genes in AMD and PCV

14

Moeen Riaz and Paul N. Baird

Abstract

Age-related macular degeneration (AMD) is a neurodegenerative disease and a cause of progressive blindness effecting millions of elderly people (over age of 50 years) worldwide. It is a complex disease with differing epidemiology, clinical features, pathogenesis, treatment, and genetics in Europeans and Asians. Specifically, polypoidal choroidal vasculopathy (PCV), a subtype of neovascular AMD, is more common in Asians compared to Western counterparts and exhibits different characteristics in all of these features.

Since the first genome-wide association study (GWAS) in 2005, which led to the discovery of the complement factor H (*CFH*) gene as a major risk factor in AMD, a number of studies have reported association of genes in the complement pathway, indicating its key role in AMD susceptibility. In addition, common genetic variants associated with AMD have been extensively investigated by two consortia, the International AMD Gene Consortium and the Genetics of AMD in Asians (GAMA), where GWAS has been undertaken on both large European and Asian AMD cohorts, respectively. These studies reported different genetic risk factors associated with AMD in Europeans and Asians.

In the case of PCV, it has been shown that a number of common genetic risk factors are shared with neovascular AMD but have different clinical presentations, perhaps indicating involvement of differential biological mechanisms, for which separate GWAS studies in large PCV cohorts are warranted.

Currently the standard treatment for neovascular AMD, as well as more recently for PCV, is anti-vascular endothelial growth factor (anti-VEGF) injections. However, different treatment outcomes have been reported and the reason for this is likely to be partly explained by genetic variants. While much progress has already been made, there are still many

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challenges that remain in understanding both AMD and PCV, in terms of early diagnosis, disease prediction, and personalized treatment approaches.

Keywords

Age-related macular degeneration • Neovascular AMD or choroidal neovascularization • Geographic Atrophy • Polypoidal choroidal vasculopathy • Genome-wide association study • Anti-vascular endothelial growth factor • International AMD Gene Consortium • Genetics of AMD in Asians Consortium • Angiogenesis • Single nucleotide polymorphisms • Ranibizumab • Bevacizumab • Visual acuity • Pharmacogenetic • Pooled GWAS • Anti-VEGF treatment response

14.1 Disease Background

14.1.1 Age-Related Macular Degeneration (AMD)

Age-related macular degeneration (AMD) presents as a progressive neurodegenerative eye disease which leads to severe visual impairment in aged people. It is most prevalent in Western populations with 1.75 million individuals affected (either with geographic atrophy or neovascular AMD) in the USA [1, 2]. It is a complex disease and its risk factors, clinical presentation, epidemiology, and treatment have been shown to differ among ethnic groups [3]. The precise etiology of AMD is unknown, but smoking, age, and genetic factors are major risk factors for AMD predisposition [4]. In 2005, the first genome-wide association study (GWAS) was conducted on AMD, identifying the association of a genetic variant in the complement factor H (*CFH*) gene with AMD [5].

Visual pathological changes in AMD begin with deposition of drusen, consisting of glycolipids, cellular debris, and proteins that reside between Bruch's membrane and the retinal pigment epithelial (RPE) cell layer of the retina. These are associated with increasing likelihood of degeneration of the macula, ultimately leading to central vision loss and potentially legal blindness [6]. The presence of small size drusen (hard drusen), typically associated with normal aging, has also been associated with risk of early AMD, but this risk tends to be conferred with the presence of multiple hard drusen within the macula [6]. In the case of soft drusen, these are typically classified as small (>63–125 μm) or intermediate (>125μm) drusen, with increasing size associated with increased risk of AMD. Additionally, the presence of hyper- or hypopigmentary changes in the RPE and thickening of Bruch's membrane all lead to increasing risk associated with the early forms of AMD [7]. The progressive accumulation of drusen, together with age and other risk factors, will ultimately lead to many individuals with early AMD progressing to the later blinding stages of the disease, referred to as either geographic atrophy (dry) AMD or neovascular (wet) AMD. The dry form is characterized by the degeneration of overlying photoreceptors and is responsible for central blindness [8], whereas neovascular AMD, although less common, is the main cause of vision loss in AMD patients. Neovascular AMD begins with leakage of fluid from newly formed blood vessels under the RPE due to abnormal angiogenesis, thereby resulting in retinal scarring that subsequently leads to loss of central vision [9, 10]. Neovascular AMD can also be further subdivided into various clinical subtypes including occult or classic choroidal neovascularization (CNV), retinal angiomatous proliferation (RAP), and polypoidal choroidal vasculopathy (PCV).

14.1.2 Polypoidal Choroidal Vasculopathy (PCV)

PCV represents a type of intrachoroidal neovascularization. It was first reported 30 years ago as an abnormal subretinal and hemorrhagic disease in the posterior pole [11]. Since then, advancements in retinal imaging, including through the use of the fluorescent dye indocyanine green angiography (ICGA), have now become a gold standard diagnostic method for PCV, allowing it to be distinguished from late AMD. For the ICGA test, indocyanine green fluorescent dye is used because it does not leak from the choriocapillaris. It has a high protein binding affinity and infrared light penetrates the RPE more efficiently than the green light emitted by the fluorescein [12]. Using this test, effective diagnosis of PCV allows visualization of early-phase nodular hyperfluorescence from the abnormal choroidal vasculature – grape like cluster formation of polyps in the inner choroid, recurrent hemorrhage, retinal pigment epithelium detachment, and absence of soft drusen [13–17]. Clinically, PCV presents as type 1 "polypoidal PCV" and type 2 "typical PCV," based on the presence or absence of branching vascular networks (BVNs), respectively. For diagnosis, 1 min after ICGA injection, type 1 PCV exhibits apparent BVNs, while type 2 PCV shows no or faint BNVs [18]. Similarly, different studies have demonstrated that both angiographic types of PCV (type 1 and type 2) manifest differences in genetic susceptibility, clinical course, and response to therapy. For instance, clinical difference between these types is that type 1 PCV has smaller polyp size, smaller choroidal thickness, and larger lesion size compared to typical PCV [19–21]. Thus clinically, it would appear that PCV is distinctly separate from AMD.

14.2 Prevalence

14.2.1 AMD

The epidemiology of AMD varies among different ethnicities around the world. It is most common in developed countries, affecting approximately 30–35 million people worldwide [22]. In the USA, the prevalence of AMD has been reported as 13.4% of 60 years or older, while in Australia, this has been reported as 14% of individuals over 50 years of age [23, 24]. It is estimated that by 2040 the number of AMD patients will be 288 million, reflecting the aging population and increased life expectancy. In Europeans, the prevalence of any AMD is 5% higher than that of Asians [25]. However, as Asia constitutes 60% of the world's population, the relative proportion of AMD patients will be high. Additionally, as Asia consists of 50 independent countries with varied ethnic groups, this leads to wide variation in reported prevalence of AMD, ranging from 2.5% to 16.1% for early AMD and 0.2% to 1.0% for late AMD [26–36]. The highest prevalence in Asia was observed in Japan and mainland China, with early AMD being 16.1% and late AMD being 1.0% [30, 37]. Cheung et al. (2012) [38] conducted prevalence studies on 10,000 AMD patients consisting of three ethnic groups, Chinese, Indians, and Malaysians and found similar prevalence rates of early AMD in individuals from China and India, while prevalence was lower in Malays. The prevalence of late AMD was similar for all three groups. On the other hand, the prevalence of AMD is low in many other Asian countries such as Pakistan, where macular degeneration accounts for only 2.1% of all blindness [39]. Few studies have investigated the incidence of AMD in Asia. The longest incidence study so far has been reported by Hisayama, which lasted 9 years and followed 1775 Japanese AMD patients with an incidence rate of 10% for early and 1.4% for late AMD individuals $\geq$ 40 years of age [40].

14.2.2 PCV

PCV can occur at a much earlier age compared to AMD, with it occurring at an age range of 21–93 years, but is most commonly diagnosed above the age of 65 years. Prevalence of PCV has been estimated by different clinical based cross-sectional studies ranging from 22.3% to 54.7% in Asian populations and 6–10% in

Caucasians, initially presumed as neovascular AMD [41, 42]. Maruko et al. (2007) [43] reported the highest prevalence of PCV, being 54.7% of neovascular AMD patients in Japan, while a report from the Singapore National Eye Centre showed 53.8% Chinese patients had PCV, which was much higher compared to two prospective studies from China which reported 22.3% [41] and 24.5% [44] patients with PCV. However, prevalence of PCV in Taiwan and Korea has been reported as 49% and 24.6%, respectively [45, 46]. Li et al. (2014) [47] reported in the Beijing eye study a prevalence of 0.3% ± 0.1% in 3468 subjects aged ≥ 50 years where ophthalmic examination, fundus photograph, and Optical Coherence Tomography (OCT) scans were undertaken. The limitation to this study was that no ICGA test was performed to diagnose PCV. Therefore it is difficult to estimate the actual prevalence of PCV in this population-based study. On the other hand, studies from Asia have reported PCV is more common in males at 63–78% compared to females, which is opposite to European studies where females are more commonly represented at 52–75% compared to males with PCV [11, 18, 48].

14.3 Risk Factors

14.3.1 AMD

AMD is a complex disease, and its multifactorial nature reflects the involvement of both environmental and genetic risk factors. The former includes age, smoking, body mass index, hypertension, obesity, cardiovascular events, UV exposure, gender, liver cancer, hepatitis B, and diet [34, 49–52]. However, only age and smoking appear consistently associated with the disease in both Caucasians and Asians. Ocular risk factors associated with AMD are not consistent among Caucasians and Asians, such as previous cataract surgery, which showed association in Europeans but did not replicate in Asians [53], while hyperopic refraction was found to be associated with AMD in both populations [50, 54]. Studies also investigated serum biomarkers such as C-reactive protein, matrix metalloproteinases, and homocysteine as risk factors for AMD and found inconsistent results [55–60]. Genetic variant associations in AMD have been extensively studied in Europeans and more recently in East Asia. In the next section of this chapter, we detail the role of genetics in AMD.

14.3.2 PCV

PCV shares some common environmental and genetic risk factors with neovascular AMD [53] with smoking being consistent. Kikuchi et al. (2007) [60] conducted a study on Chinese PCV patients and reported subjects who smoked had a four fold increased risk of PCV when compared with age- and gender-matched controls ($P < 0.001$, odds ratio (OR) 4.4 95%, CI 2.5–7.7). However, ocular risk factors like cataract surgery and glaucoma were not associated with PCV susceptibility [61]. Chung et al. (2011) [62] performed enhanced depth imaging (EDI) with spectral domain (SD)-OCT and found a high choroidal thickness in PCV patients ($p < 0.0001$), but thinner choroidal thickness in neovascular AMD ($P = 0.0004$) compared to controls. This has been replicated in other studies [63–65] which indicates the measurement of choroidal thickness by SD-OCT may provide a useful clinical biomarker to distinguish between neovascular AMD and PCV. Other sources of biomarkers such as in serum have so far been inconsistent. For example, increased serum levels of MMP9 did not appear to differentiate between neovascular AMD and PCV, while another study reported increased MMP9 and MMP2 serum levels in PCV but not in neovascular AMD [57, 58]. Genetic variants appear to play a major role in PCV pathogenesis which is discussed in the next section of this chapter.

14.4 Genetics Studies

14.4.1 AMD

Multiple association studies have been conducted in AMD through the screening of various genetic markers termed single nucleotide polymorphisms

(SNPs), as well as searching for structural variants such as copy number variations (CNVs). In the next section, details of genetic involvement and their role in AMD pathogenesis are described.

14.4.1.1 Linkage Studies

The role of genetics in AMD has been extensively investigated since initial studies in the 1990s observed increased familial aggregation of disease [66], as well as a higher concordance of disease in identical twins compared to non-identical twins [67]. In 1998, Klein et al. used linkage studies to identify a locus at chromosome 1q25-q31 that was linked to dry AMD in a family of ten affected members [68]. Subsequently, it led to several AMD family-based linkage studies which successfully implicated a number of genomic regions harboring susceptibility loci for AMD [69–73]. However, linkage studies for AMD have been limited by the lack of multigenerational families due to the late onset of the disease, the tendency for smaller families with less affected sib-pairs, and phenotypic heterogeneity.

14.4.1.2 Candidate Gene Studies

As a response to the limited availability of AMD families together with changes in genetic technology, other approaches were investigated. One such approach was to perform association studies by screening genetic markers such as SNPs and CNVs in candidate genes identified as associated with other macula diseases that shared similar pathological features with AMD. These included the screening of genes such as the *RDS* gene for Retinitis pigmentosa [74], the *EFEMP1* for Doyne honeycomb retinal dystrophy [75], the *VMD2* gene for Best's macular dystrophy [76], the *TIPM3* gene for Sorsby's fundus dystrophy [77], the *ELOVLA4* for Stargardt-like macular dystrophy [78], and the *ABC4* gene for Stargardt's disease [79]. While the *ABC4* gene was initially shown to be associated with AMD, other reports were negative resulting in the conclusion that this gene was not a major player contributing to AMD pathology [80] but more likely associated with a subgroup of AMD [81]. A third approach has been to perform case control association studies and screen candidate gene that have functional roles in other neurodegenerative disorders, or lipid and cholesterol disorders, such as the association of the Apolipoprotein E (*APOE*) gene with AMD. As its name suggests, the *APOE* gene is a lipid transport protein and involved in the transportation of cholesterol and lipoproteins into the blood through the lymph system [82]. Furthermore Baird et al. (2006) [83] reported the association of the $\varepsilon 2$ allele with progression of AMD in females, indicating a gender-specific role of the *APOE* gene in AMD.

14.4.1.3 Genome-Wide Association Studies

With the advancement of genetic techniques, Klein et al. (2005) [5] performed the first ever successful genome-wide association study (GWAS), using only 96 AMD cases and 50 non disease controls, where the SNP rs1061170 (Y402H) of the complement factor H *(CFH)* gene was identified as a strong genetic risk factor associated with AMD. Later on, this finding was replicated in many independent studies in both Caucasians and Asians. However, some studies reported no association of rs1061170 in Asian populations [84–86] indicating that ethnic differences may be present. This finding was confirmed by the AMD Gene Consortium which showed that the Y402H variant was more strongly associated with European populations compared to Asian populations [87]. The *CFH* gene encodes a protein known as complement factor H, comprising 1213 amino acids arranged in 20 homologous units, each consisting of approximately 60 amino acids and functions as an inhibitor of the inflammatory response. The anti-inflammatory C-reactive protein (CRP) which has also been found to be associated with progression of AMD [88] binds with CFH and is thought to impact on its role in the alternative pathway (AP). In this pathway, CFH breaks the complex between C3b and factor B and converts C3b into its inactive form C3bi, thus inhibiting the inflammatory response [89].

Reports on strong association of the *CFH* gene with AMD suggested an important role of

the complement pathway in AMD pathogenesis. This subsequently led to identification of several other complement genes such *C3*, *C2*/*CFB*, *C9*, and *CFI* as associated with AMD, indicating the important role that immunity plays in AMD [90–95]. The association of two SNPs rs1047286 and rs2230199 in the *C3* gene with AMD was consistent with other European studies [96–98]. In contrast, no association has been reported for the *C3* gene polymorphisms (rs2230199 and rs1047286) with AMD in Chinese where a lower minor allele frequency (<5%) of these SNPs was present [84]. Later, Thakkinstian et al. (2011) [91] performed a meta-analysis of 23 studies on Asians and Caucasians, consisting of 12,770 and 8118 subjects and reported significant association of *C3* gene polymorphisms (rs2230199 and rs1047286) with AMD ($p < 0.001$) in Caucasians but not in Asians ($P > 0.05$), suggesting genetic difference in populations.

When considering AMD prevalence in various ethnic populations, it does not necessarily correlate with the frequency of the C risk allele of SNP rs1061170 of the *CFH* gene. For instance, the frequency of this allele has been reported at approximately 7% in the Japanese compared to 34% in Caucasians, but the prevalence of AMD in the Japanese is approximately half of that compared to Caucasians (12.7 vs 28%) [31, 99, 100].

14.4.1.4 Non-chromosome 1 Loci and AMD

While the alternative complement pathway appears to play a key role in AMD, genome-wide linkage scans of extended families and case control association studies also consistently reported a strong signal at chromosome 10q26 associated with AMD susceptibility [101]. This region contains two neighbouring genes, *LOC387715*/*ARMS2* and *HTRA1*, which are both in high linkage disequilibrium (LD). Subsequently, many independent studies have reported association of variants in both of these genes with AMD, in both Asians and Caucasians [94, 102, 103]. The functional variant, rs11200638, in the promoter region of the *HTRA1* gene was associated with increased risk of AMD in Caucasians ($p = 5.9 \times 10^{-8}$; odds ratio = 2.3), and similar results have been reported in Chinese populations ($p = 8.2 \times 10^{-12}$; odds ratio = 1.6) [102, 104]. The SNP rs10490924, near the *ARMS2* gene, is in high LD with rs11200638 ($D^2 > 0.99$) and is also associated with AMD in both Caucasian and Asian populations [105]. Dissecting which of these two genes may contain a causative variant has so far proved elusive with high levels of *HTRA1* mRNA reported in lymphocytes of AMD patients with a population attributable risk of 49.3% for rs11200638 [104]. In contrast, Fritsche et al. (2008) [106] suggested *ARMS2* was a stronger candidate gene for AMD because polymorphisms in this gene lead to expression of an ARMS2 isoform which is unstable at the mRNA level and ultimately leads to no protein expression. Similarly, studies in mainland northern Chinese have shown association of both rs11200638 and rs10490924 in neovascular AMD, but following conditioning on rs11200638, only the *AMRS2* variant rs10490924 remained associated with neovascular AMD ($p < 0.001$). An in vitro study model of RF/6A and RPE cells confirmed expression of the rs10490924 A69S mutated ARMS2 mRNA that leads to increased proliferation and migration of cells compared to the wild-type ARMS2 ($p < 0.01$). However, RF/6A cells with A69S mutant and wild-type form of this variant showed no significant difference in forming tube networks ($p > 0.05$) compared to controls [107]. Kanda et al. (2007) [108] reported expression of ARMS2 protein localized to the mitochondrial outer membrane in transfected mammalian cells, indicating a mechanism by which A69S might be involved in AMD pathogenesis. However, mammalian cells did not show any significant difference in expression and stability of the A69S ARMS2 protein compared to wild type. Later on, the same group confirmed expression of ARMS2 protein in mitochondria in rod and cone inner segments of human retina. They proposed a functional role for the ARMS2 protein in mitochondrial homeostasis but also stated that the exact function of this protein in AMD pathogenesis was still to be

elucidated [106]. Thus, this region on chromosome 10 is still to be resolved and represents an unanswered question in the aetiology of AMD.

14.4.1.5 Lipid and Other Pathway Genes

A number of other genes involved in lipid metabolism also appear to be associated with AMD pathogenesis. These include *LIPC*, *APOE*, *CETP*, *ABCA1*, and *LPL* [93, 109, 110]. The *APOE* gene functions in lipoprotein transportation and is expressed in Bruch's membrane, outer segments of photoreceptors, and the ganglion cell layer in the retina. It presents with three isoforms resulting from the different allelic variant combinations, $\varepsilon 2$, $\varepsilon 3$ and $\varepsilon 4$, with the latter being consistently reported as protective for AMD ($p = 4.4 \times 10^{-11}$, OR = 0.72), while the $\varepsilon 2$ being significantly associated with increased AMD risk ($p = 4.0 \times 10^{-2}$, OR = 1.83) [111]. In addition, genes in the angiogenesis pathway are also associated with AMD, such as the *VEGF* gene in both Caucasians and Asians. This gene is expressed in the retina with higher levels in AMD patients considered as a major factor in initiating choroidal neovascularization (CNV) [93] and has provided the impetus for most of the current anti-angiogenic treatments in existence today.

Importantly, rapid identification of additional common variants in AMD has been aided by a global network of research investigators representing 18 research groups from around the globe (the AMD Gene Consortium). This consortium undertook a GWAS meta-analysis on the largest cohort of 17,100 AMD patients and 60,000 controls so far collected for this disease and reported the identification of 19 loci. Twelve of these had been previously reported at the genome-wide significance level by numerous studies, but an additional seven loci were reported for the first time near the genes: *RAD51B*, *TGFBR1*, *B3GATL*, *SLC16A8*, *IER3-DDR1*, *ADAMTS9*, and *COL8A1*. Interestingly, subgroup analysis of neovascular AMD and GA showed independent association with *ARMS2* ($p = 8.0 \times 10^{-4}$) and *CFH* ($p = 6.0 \times 10^{-4}$) genes, respectively. Following stratification by ancestry showed variable effect sizes, such as variants near the *CFH* gene were more strongly associated in Europeans ($p = 1 \times 10^{-7}$) compared to East Asians, whereas SNPs near the *TNFRSF10A* gene showed the strongest association in Asians compared to Europeans ($p = 2.0 \times 10^{-3}$) [87]. Spurred on by this endeavor, a second consortium "the International AMD Genomics Consortium" has been established to investigate other genetic variants including rare variants in AMD. Recently, Fritsche et al. (2015) [112] compared the 1000 genome project data of 379 Europeans and 286 East Asians to determine the effect of allele frequencies of known AMD genetic risk variant loci among different populations. Comparison of the *CFH* gene variant rs10737680 showed a similar allele frequency among Europeans and Asians, while SNP rs2230199 in the *C3* gene was common in Europeans (20.4%) but rare in Asians (0.3%). In contrast, the SNP rs10490924 was present at 40% in East Asians compared to only 20% in Europeans, suggesting it likely plays an important role in AMD susceptibility. These findings further indicate the diversity of AMD genetics in different populations.

14.4.1.6 East Asian AMD GWAS

The Genetics of AMD in Asians (GAMA) Consortium has also been established by research investigators from East Asia with the aim of uncovering genetic involvement in AMD patients of Asian ancestry. This Consortium recently reported a GWAS and exome-wide association study on 6345 neovascular AMD cases and 15,980 controls. Their main findings were four novel loci: *CETP* (rs2303790), *C6orf223* (rs2295334), *SLC44A4* (rs12661281), and *FGD6* (rs10507047) associated with an increased risk of AMD in East Asian patients. In contrast to the 19 loci reported by the AMD Gene Consortium, only nine of these were replicated in the East Asian study, *APOE* (rs4420638), *ADAMTS9* (rs6795735), *ARMS2-HTRA1* (rs10490924), *VEGFA* (rs943080), *CFH* (rs10737680), *CETP* (rs3764261), *C2-CFB* (rs429608), *CFI* (rs4698775), and *TGFBR1* (rs334353). Interestingly, the *CETP* 442G

variant (odds ratio (OR) = 1.73, $p = 5.60 \times 10^{-16}$) was only present in East Asians (controls MAF <5%) and not Europeans. This SNP is a coding variant and known to impair *CETP* protein function. It plays a major role in regulating high-density lipoproteins cholesterol (HDL-C), suggesting a vital role of lipid metabolism in AMD [113]. Importantly, while the findings suggest some similarity in East Asians with Europeans, it is likely that East Asians also have a distinct genetic signature.

Another recent study performed whole exome sequencing on 216 Chinese neovascular AMD cases and 1553 controls followed by replication in six independent AMD cohorts (3988 cases and 8495 controls) from East Asia. A non-synonymous SNP rs7739323 (V379M) in the *UBE3D* gene showed association with AMD ($p = 1.16 \times 10^{-9}$). The *UBE3D* gene encodes ubiquitin ligase E3 protein and functions in the cellular ubiquitin-proteasome system (UPS), which is vital for regulating the sorting and degradation of intracellular proteins. To determine the functional role of rs7739323 on the *UBE3D* protein and its associated link to AMD, they used clustered regularly inter-spaced short palindromic repeats (CRISPR) gene editing technology to generate $UBE3D^{-/-}$ knockout mice, which did not survive, but the heterozygous $UBE3D^{+/-}$ mice survived. This suggested that the *UBE3D* gene plays an important role in mouse development. Electron microscopy of heterozygous $UB3ED^{+/-}$ knockout mouse retina showed deposits of pigment granules and loose retinal pigment villi when compared with control mice. In addition, $UBE3D^{+/-}$ mice also showed declined electroretinography (ERG) responses. Thus, dysfunctional UBE3D protein leads to structural changes in mice retina and abnormal ERG in $UBE3D^{+/-}$ mice indicating a role of UPS in AMD pathogenesis [114].

14.4.1.7 Rare Genetic Variant Association Studies

While most GWAS studies have been focused on the search for common genetic variants with an allele frequency typically in excess of >5%, another source of genetic variation has also been proposed. This comes from rare genetic variants (typically with a minor allele frequency <1%) but where a large odd ratio associated with risk of disease has been predicted. Several such studies have now been undertaken in European populations. These studies have concentrated on the known complement genes associated with AMD and typically involve targeted gene sequencing in many thousands of individuals. For instance, Seddon et al. (2013) [115] performed a screen of 5115 AMD individuals for 681 genes (reported AMD loci) and found association of rare variants (allele frequency of <1%) p.lys155Gln ($P = 2.0 \times 10^{-6}$, $OR = 16.7$) in the *C3* gene and p.Pro167Ser ($P = 2.4 \times 10^{-5}$; OR = 2.2) in the *C9* gene, thus confirming the large effect size of such variants. They also tested the gene burden of rare variants in cases compared to controls and found 7.8% of cases had rare variants in the *CFI* gene compared to 2.3% in controls (OR = 3.6, $P = 2 \times 10^{-8}$). In parallel, the rare variant R1210C in the *CFH* gene ($p = 2.9 \times 10^{-6}$, OR = 23.11) and p.Lys155Gln encoded in the C3 gene ($p = 2.7 \times 10^{-4}$; OR = 2.68) were described as associated with AMD [116]. Similarly, a recent study from Kavanagh et al. (2015) [117] reported that rare variants in the *CFI* gene were associated with AMD. In addition, individuals with lower levels of factor I protein in serum and the rare variant p.A240G in the *CFI* gene are more likely to have advanced AMD compared to patients without this rare variant ($P = 5.1 \times 10^{-4}$, OR 7.2) and when compared to control individuals with no rare variant ($P = 1.1 \times 10^{-5}$, OR 19.0). Importantly, CFI is a serum serine protease that cleaves C3b with the help of its cofactors and plays a major role in activation of the alternative pathway, thereby impacting on the functional capabilities of the complement activation.

14.4.2 PCV

Few GWAS have been conducted for PCV with most genetic studies being based on assessment of known AMD associated loci. Initially, the two major AMD susceptible genes *CFH* and *ARM2*/

HTRA1 were assessed for association with PCV. Firstly, association of SNPs in the *CFH* gene has shown inconsistent results in Caucasian and Asian PCV populations. For instance, association with rs1061170 ($p = 0.0002$) was described in European PCV patients but was based on a small sample size of only 45 individuals [118]. Similarly, a study from Japan with 518 PCV patients confirmed association of the two SNPs rs1061170 (Y402H) and rs800292 (I62V) in the *CFH* gene when compared to 1351 control individuals ($P = 1.54 \times 10^{-6}$; I62V, $P = 1.94 \times 10^{-29}$, respectively) [119]. However, another study from Japan, while reporting significant association of the I62V polymorphism with PCV ($P = 1.54 \times 10^{-6}$), failed to show association with rs1061170 ($p > 0.05$) [120]. Additionally, Sakurada et al. (2011) [121] reported no association of I62V with clinical phenotypes of PCV ($p > 0.05$). Variable association results could be due to ethnic genetic differences, such as for the rs1061170 C risk allele, as its frequency is very low in Asians (11.9% Chinese and 7% Japanese) compared to Caucasians (39.4%) [99, 100]. Secondly, significant association of SNPs in the *ARMS2*/*HTRA1* gene region has been shown with PCV [105, 119, 121–124]. A report on 109 Japanese PCV patients demonstrated association of rs10490924 with PCV ($P < 0.0001$) with an increased risk of 8.4-fold for the TT genotype compared to the wild-type GG genotype [123]. Another Japanese study corroborated the association of rs10490924 *ARMS2* and rs11200638 in the *HTRA1* gene using 243 individuals (73 neovascular AMD, 76 PCV, and 94 controls). They found association of both SNPs with PCV (rs11200638 $P = 5.2 \times 10^{-6}$, OR = 6.3, 95% CI= 2.67, 16.03) and rs10490924 ($P = 5.7 \times 10^{-6}$, OR = 6.54, 95% CI = 2.67, 16.03), as well as with neovascular AMD (rs11200638, $P = 3.4 \times 10^{-7}$, OR = 13.7, 95% CI = 4.52, 42.02) and rs10490924 ($P = 1.4 \times 10^{-6}$, OR = 11.4, 95% CI = 3.92, 33.11), respectively. This study confirmed that effect sizes of both SNPs are higher in AMD cases compared to PCV, which implies that other genes might be involved in susceptibility of PCV in the Japanese population [105]. Similarly, studies in mainland northern Chinese have shown association of both rs11200638 and rs10490924 in PCV, but following conditioning on rs11200638, only the *AMRS2* variant rs10490924 remained associated with PCV ($p < 0.001$) [124]. Furthermore, Li et al. (2015) [125] performed a comprehensive systematic meta-analysis on 66 published reports on the genetics of PCV and found strong association of two SNPs in the *CFH* gene region, rs1061170 ($P = 3.36 \times 10^{-11}$, OR = 1.76, 95% CI, 1.49–2.09) and rs800292 ($P = 2.66 \times 10^{-56}$, OR = 0.51, 95% CI, 0.47–0.55), and two SNPs rs10490924 ($P = 6.61 \times 10^{-30}$, OR = 2.44, 95% CI, 2.09–2.84) and rs11200638 ($P = 1.76 \times 10^{-42}$, OR = 2.36, 95% CI, 2.08–2.67) in the *ARMS*/*HTRA1* region. In addition, SNP rs10490924 was the most frequently investigated SNP in PCV. Interestingly, Sakurada et al. (2008) [123] reported that the *ARMS2* SNP rs10490924 is also associated with more severe clinical manifestation of PCV in the Japanese, such that 88.9% of PCV patients with vitreous hemorrhage had the T risk allele compared to 37% of PCV subjects without vitreous hemorrhage ($p = 0.009$). This was later corroborated by the same group using a larger cohort of 226 PCV patients. Here, they demonstrated that subjects with homozygous TT genotypes for rs10490924 had a 12-fold increased chance of having hemorrhagic pigment epithelial detachment (PED) ($P = 0.0001$), early onset of disease (68.8 years, $P = 0.02$), and more likely to have bilateral disease ($P = 0.007$), compared to individuals with the wild-type GG genotype [121]. From this it can be speculated that genotyping of rs10490924 will be useful for gleaning a better understanding of the clinical characteristics of PCV.

A number of other genetic associations have been reported in one or two studies with PCV including those of *APOE* [126], *ELN* [127], *PEDF* [128], *TLR3* [111], *SOD2* [129], *RDBP* [130], *SKIV2L* [130], *9p21* [131], *8p21* [85], *4q12* [132], *C2*/*CFB* [118, 120], *C3* [122], *VEGFA* [133], and *LIPC* [110]. However, a

recent detailed meta-analysis reported association of the variants *CFB* rs4151657, *RDBP* rs3880457, *C2* rs547154 *SKIV2L* rs429608 and rs2075702, *CETP* rs3764261, *8p21* rs13278062, and *4q12* rs1713985, showing the most consistent significant association with PCV [125]. In addition, variants in eight genes, *ELN*, *LIPC*, *LPL*, *ABCA1*, *VEGFA*, *TLR3*, *LOXL1*, and *SERPING1*, did not appear associated with PCV. These studies suggest the genetic architecture of PCV has still not been fully explored and will require the use of larger cohort studies using GWAS and exome or whole genome sequencing to uncover more variants.

14.4.2.1 GWAS in PCV

A small GWAS study was conducted on 100 PCV patients, 100 neovascular AMD patients, and 190 age-matched control population and two variants reported, rs10490924 ($P = 3.7 \times 10–^{8}$, OR = 2.72) of *ARMS2* and *rs800292* ($P = 2.6 \times 10^{-4}$, OR = 2.00) of the *CFH gene*, associated with both neovascular AMD and PCV. In the case of SNP rs2241394 ($P = 2.5 \times 10^{-3}$, OR 3.47) of the *C3* gene, this showed association only with PCV when compared with both AMD and control populations [134].

A much larger GWAS has been undertaken to better identify genetic variants in PCV through the GAMA Consortium study (see section on AMD genetics). This study on 2119 patients with exudative AMD and 5691 controls, followed by independent replication in 4226 patients and 10,289 controls, was undertaken on samples from Singapore, China, Hong Kong, and Japan. It identified four novel SNPs, in particular, the *CETP* gene polymorphism rs2303790 (specific in East Asians) with neovascular AMD as well as the Ala231Ala change in the *C6orf223* gene (OR = 0.78, P=6.19 $\times$ 10^{-18}), the Asp47Val change in the *SLC44A4* gene (OR = 1.27, $P = 1.08 \times 10^{-11}$), and the Gln257Arg change in the *FGD6* gene (OR=0.87, P=2.85 $\times$ 10^{-8}). Subgroup analysis of these top four variants with either neovascular AMD (n = 1083) or PCV (n = 1015) cases indicated similar levels of association and effect size [113]. This would seemingly imply that genes identified as associated with AMD are also associated with PCV. However, it did not indicate genome-wide associations of novel genes associated with PCV that are independent of AMD-associated genes. In the case of the *CETP* gene, there are several studies that have reported association of variants in this gene with PCV [135–137]. Interestingly, recent meta-analysis on 1058 PCV cases and 1272 controls found significant association of rs3764261 ($P = 2.22\times10^{-7}$, 95% CI, 1.27–1.69, OR = 1.46) with PCV [125].

14.5 Treatment

14.5.1 AMD

14.5.1.1 Geographic Atrophy (GA)

The GA (dry) disease subtype accounts for 85% of AMD patients, but yet effective treatment and management continues to be a challenge. However, a number of clinical trials are ongoing such as the phase III trial of lampalizumab (Genentech/Roche, South San Francisco, CA, USA) which targets GA patients with an antigen-binding fragment of a humanized, monoclonal antibody against complement factor D to slow progression of GA. This investigational drug inhibits complement factor D and blocks activation of the alternative complement pathway. The phase II trial MAHALO study reported that monthly intravitreal injection of lampalizumab (anti-factor D) in 129 bilateral GA patients showed a 20% reduction in growth rate of GA atrophy. More interestingly patients with a specific mutation in *CFI* gene showed a 44% reduction in GA atrophy, which would represent one of the first uses of precision medicine in AMD [138].

14.5.1.2 Choroidal Neovascular Disease (CNV)

In the remaining 15% of patients who present with the neovascular (CNV or wet) form of AMD, there have been a number of different treatment options. The first effective treatment

introduced to halt neovascular AMD was laser photocoagulation therapy [139, 140], but due to the risk of loss of visual acuity (VA) and recurrent neovascularization, it was replaced by photodynamic therapy with Verteporfin (Visudyne™, Novartis, Basel, Switzerland) [141] and later with the more efficient anti-VEGF agents [142]. Currently, the most suitable treatment for neovascular AMD is recombinant, humanized anti-VEFG monoclonal antibody drugs such as ranibizumab (Lucentis®), bevacizumab (Avastin®), aflibercept (Eylea®), [142–145], and more recently the development of conbercept (KH902; Chengdu Kanghong Biotech Co., Ltd., Sichuan, China) [146]. While these anti-VEGF therapies have been particularly useful at treating the neovascularization, Rofagha et al. (2013) [147] reported a 7-year multi-center observational study update of macular degeneration patients who were treated in the original post-MARINA/ANCHOR and HORIZON trials (SEVEN UP). This study showed that after an average of 7.3 years of Lucentis treatment in neovascular AMD patients, approximately 50% of patients improved or maintained their vision (> 0–1 Early Treatment Diabetic Retinopathy Study (ETDRS) letters). However, in the other half of patients, there was an overall loss of 8.2 ETDRS letters VA indicating that anti-VEGF treatment may have limitations as an effective long-term treatment strategy. A number of studies have also compared ranibizumab and bevacizumab efficacy, including the 2-year Comparison of AMD Treatment Trial (CATT) study and Inhibit VEGF in Age-related choroidal Neovascularization (IVAN). Both of these studies demonstrated that Lucentis and Avastin showed an equivalent response (non-inferior) in VA improvement with an overall gain of 8.8 and 7.8 ETDRS letters, respectively [148]. In addition, Eylea, a relatively new anti-VEGF drug approved following the two clinical trials, VEGF Trap-Eye: Investigation of Efficacy and Safety in Wet AMD (VIEW 1) and VIEW 2, both demonstrated that after an initial three monthly injections of Eylea followed by subsequent injections every 2 months, was non-inferior to Lucentis monthly treatment in AMD treatment-naive patients [149]. A number of studies have also assessed short-term outcomes of Eylea given to already Lucentis treatment-resistant AMD patients and confirmed that improvements in visual acuity (VA) and anatomical functions can be obtained through such sequential treatment [150, 151].

14.5.2 PCV

For PCV, the treatment options are laser photocoagulation [152], photodynamic therapy (PDT) [152], anti-VEGF therapy [153], and combination therapy (PDT and anti-VEGF agents) [154]. Initially, laser photocoagulation was the only treatment option, which was later replaced with PDT due to its better efficacy and, then more recently, anti-VEGF agents. However, laser photocoagulation is still a treatment option for extrafoveal PCV. Cheung et al. (2014) [155] treated 31 PCV patients with thermal laser therapy and reported 90.3% patients improved or stabilized their vision at 12 months.

In the case of PDT, the EVEREST (efficacy and safety of verteporfin photodynamic therapy in combination with ranibizumab, or alone, versus ranibizumab monotherapy in patients with symptomatic macular polypoidal choroidal vasculopathy) study was the first randomized control trial to evaluate PDT with or without ranibizumab. This study reported PCV monotherapy with PDT gave high polyp closure (71%) compared to ranibizumab (Lucentis) (28.6%) but showed no difference in VA improvement [154], while the multicenter LAP-TOP study trial showed an improvement of 0.2logMAR in VA in 30% of PCV patients ($P = 0.03$) treated with ranibizumab alone compared to PDT monotherapy [156]. However, these studies used anti-VEGF agents as adjunct therapy. Later, several studies have reported the use of anti-VEGF agents as monotherapy in treatment-naive PCV patients [153, 154, 156–159].

Hikichi et al. (2013) [158] reported longer-term efficacy of ranibizumab treatment in PCV. In this study, a total of 75 PCV patients were

treated with three monthly intravitreal ranibizumab injections, followed by an "as needed injection" protocol for up to 24 months. They found that vision improved with mean VA of 0.37 Logarithm Of Minimal Angle Of Resolution (logMAR) at 12 month and 0.41logMAR at 24 months compared to a baseline VA of 0.59logMAR. Complete polyp regression was observed in 40% of patients at 12 months but reduced to 25% patients at the 24-month time point. Despite the promising improvement in visual outcome, the branching vascular network persisted and increased over the 2-year ranibizumab treatment period. Similarly, Lai et al. (2008) [160] reported that all PCV patients who had persistent polyps after three monthly bevacizumab injections showed a mean VA improvement of 0.51logMAR compared to a baseline of 0.62logMAR ($P = 0.01$). A study of the use of ranibizumab or bevacizumab in Koreans with PCV was shown to have a similar efficacy for treatment outcome in improvement of visual acuity, central macular thickness, and polyp regression at 6 months of therapy [157]. However, Cho et al. (2012) [161], in a study of 60 patients treated with ranibizumab and 61 treated with bevacizumab for up to 12 months, showed no significant difference in terms of VA improvement, polyp regression, or macular thickness.

More recent studies on the use of Eylea in treatment of PCV have been limited. Short-term effectiveness (6 months) of Eylea on 13 PCV patients from Japan showed 75% polyp regression, which was higher than other studies reporting treatment outcome with Lucentis and PDT [162]. In contrast, a study of 33 PCV patients treated with Eylea reported a low polyp regression rate (48%) but an improvement of 8.5 ETDRS letters VA that was similar to the group of patients treated with Lucentis in the EVEREST study [162]. Combination treatment of PCV with Eylea and Lucentis has shown promising results, but the persistence of abnormal branching of the vasculature presents a risk of recurrence. Further studies with a longer treatment period and larger patient sample size will be required to demonstrate the long-term utility and effectiveness of anti-VEGF agents for PCV.

14.6 Pharmacogenetic Studies

14.6.1 AMD

While it is known that clinical factors such as delay to treatment and presenting visual acuity can affect response to treatment by anti-VEGFs, an individual's genetic background will also likely play a role in this response. Genetic factors have been investigated in anti-VEGF treatment response, but these have typically been the already known AMD risk-associated genes. For instance, individuals with the CC risk genotype of the SNP rs1061170 (Y402H) of the *CFH* gene have been shown to have a reduced VA improvement compared to patients with either the TC or TT genotypes when treated with Avastin [163]. In contrast, following 9 months of ranibizumab treatment, patients with the Y402H CC risk genotype showed no difference in VA response but required more injections compared to the two other genotypes (TC and TT) [164]. Association studies of rs1061170 on AMD patients from Japan showed no association after 1 year of ranibizumab treatment [165]. Recently, Chen et al. (2015) [166] performed a meta-analysis of rs1061170 from 2704 patients (13 published studies) and reported that patients with the CC genotype were less likely to respond to ranibizumab treatment compared to those with the TT genotype (OR = 0.55, 95% CI = 0.31–0.95, $P = 0.03$) or CT genotype (OR = 0.60, 95% CI, 0.40–0.91, $P = 0.02$).

In the case of the *HTRA1/ARMS2* locus at chromosome 10q26, again, studies have reported inconsistent associations of variants in this region with response to anti-VEGF treatments [167–170]. Orlin et al. (2012) [168] reported SNP rs10490924 in the *ARMS2* gene, where a high percentage of the risk allele, TT, was found in positive responders (mean improvement in VA) compared to negative responders, who lost VA, although this was not statistically significant. In contrast, Mckibbin et al. (2012) [170]

demonstrated that the SNP rs11200638 (high linkage disequilibrium with rs10490924 $r^2 > 0.8$) of the *HTRA1* was significantly associated with better VA outcome, with an overall gain of 7.5 and 2.9 ETDRS letters for the high-risk genotypes of GA and AA, respectively, compared to 2.2 ETDRS letters VA gain for the non-risk genotype GG, after 6 months of anti-VEGF treatment outcome. However, in mid-2013, Abedi et al. (2013) [171] reported significant association of both SNPs rs10200638 and rs10490924 in these genes, exhibiting poor VA response of −2.9 and −2.6 ETDRS letter loss, respectively, in AMD patients after 1 year of anti-VEGF treatment.

Genes in the VEGF pathway have also been considered as positive predictors for anti-VEGF treatment outcome. For instance, SNP rs3052000 showed an overall gain of 7 ETDRS letters after 1 year of anti-VEGF therapy in AMD [172]. Two other SNPs, rs833069 and rs3025033 in this gene, did not appear to contribute to this change in VA but were significantly associated with anatomical response of central subfield macular thickness (CSMT) and continuous leakage from neovascular AMD, respectively [173, 174]. However, a European report showed no association of tag SNPs across the *VEGF* gene or its receptor (*VEGFR2*) with VA, but did confirm that the SNPs rs6828477 and rs4576072 of the *VEGFR2* gene were associated with improved vision (gain of 15 ETDRS letters) after 1 year of ranibizumab treatment in AMD patients [175]. In contrast, Hagstrom et al. (2015) [176] reported the largest study of 1347 AMD cases combining two clinical trials (IVAN and CATT) and reported no association of the SNPs rs4576072 and rs6828477 in the *VEGFR2* gene with neovascular AMD after 1 year of either bevacizumab or ranibizumab treatment.

Rather than considering only single SNPs or genes, another approach to identify the genetic effect on anti-VEGF therapy has been to combine several SNPs. Smailhodzic et al. (2012) [177] reported the cumulative effect of risk alleles of multiple SNPs (rs699947, rs10490924, rs1061170) from the *VEGF*, *ARMS2*, and *CFH* genes, respectively. They firstly showed that patients presenting with four risk alleles from two of the SNPs (rs1061170 and rs10490924) had no improvement in VA after 1 year and that the addition of two more risk alleles from the rs699947 SNP demonstrated that carriers of six risk alleles from three SNPs showed a loss of ten ETDRS letters VA after 1 year of Lucentis treatment. In Asian AMD cohorts, there have been limited pharmacogenetic studies on anti-VEGF treatment. However, a Chinese study demonstrated that individuals presenting with the CC risk genotype of SNP rs800292 in the *CFH* gene had a mean VA of 4.4 ETDRS letters compared to 15.5 letters for those individuals with the TT genotype after bevacizumab treatment [178]. In contrast, a Korean study showed no association of the SNP rs1061170 in the *CFH* gene with either visual outcome or central macular thickness (CMT) ($p > 0.05$) after 1 year of intravitreal bevacizumab treatment [179]. A meta-analysis on rs1061170 in Caucasians versus East Asians did indicate that the CC genotype remained associated with Caucasians ($p < 0.05$) and not in Asians ($p > 0.05$) following treatment response [166]. Another study in the Korean population screened 17 SNPs in 13 AMD candidate genes and reported no association of the *HTRA1* gene polymorphism rs11200638 with VA, but it did exhibit borderline association with reduction of CMT (corrected $p = 0.052$) at 12 months of treatment. In this study, the SNP rs3025039 in the *VEGFA* was significantly associated with better visual outcome (OR = 4.57, 95% CI = 1.89–11.1, corrected $p = 0.04$) with the homozygous minor allele gaining >15 ETDRS letters VA after 1 year of ranibizumab or bevacizumab treatment [179]. On the other hand, a Japanese study showed patients with the GG genotype for SNP rs699946 in the *VEGF* gene showing a significant trend to better VA compared to individuals having the GA or AA genotypes ($p = 0.01$) after 12 months of bevacizumab treatment [180]. Further compounding the ability to dissect out the involvement of allelic variants has been the observation that ethnic differences exist across Asia. For instance, SNP rs699947 in the *VEGF*

gene has been shown to be significantly associated with poor VA response ($p = 5.11 \times 10^{-3}$) in a Japanese population, while the same SNP was not associated with change in VA in Koreans (corrected $p > 0.05$) [181, 182].

14.6.1.1 GWAS and Anti-VEGF Treatment

To date, there has only been one published GWAS case control association study undertaken to identify genetic risk factors for treatment response to neovascular AMD. This study consisted of 65 AMD patients and reported SNP rs2298515 as associated with central macular thickness (CMT) after 1 year of treatment with a genome-wide significance of (uncorrected $P = 3.85 \times 10^{-8}$)and corrected $P = 0.01$ but was not replicated. This SNP is located in an upstream region of NCRNA00158, which is a non-coding RNA gene on chromosome 21 and expresses a micro-RNA with an unknown function. The only SNP associated with poor VA outcome after 6 months of treatment was rs9675979 near the *CCDC102B* gene [173]. No GWAS has been reported for assessing anti-VEGF treatment response for PCV or neovascular AMD in Asians.

14.6.2 PCV

Pharmacogenetic studies in PCV have been undertaken to investigate the differential response of treatment such as PDT, anti-VEGF agents, and combination therapy. In a Japanese study on 93 PCV patients who underwent PDT, three SNPs in the *CFH* gene region, rs1061170 (Y402H), rs1410996, and rs800292 (I62V), were genotyped. The criteria for treatment outcome were based on anatomical measures, whereby patients who had no retinal fluid after one or two consecutive sessions of PDT were termed as responders, whereas the rest were termed nonresponders. SNPs rs1061170 and rs1410996 were significantly associated with anatomical response ($P = 0.008$ and $P = 0.011$, respectively) to PDT [183]. Another study in Japanese, examined the variant rs10490924 in the *ARMS2* gene and indicated that following PDT, individuals with the T risk allele had a significantly larger lesion size ($P = 0.00073$) and poor VA ($P = 0.014$) compared to other genotypes after 12 months of PDT [184]. A more extensive Japanese study, screened 638 SNPs across 42 - AMD-associated genetic loci in 31 treatment-naive PCV patients. Variants in four genes, *FBLN5* rs17732513, *CX3CR1* rs17793056, *SERPINF1* rs12603825, and *TLR4* rs11536889, showed significant association with PDT treatment outcome. Subsequent replication of these four variants was performed in an additional of 136 patients. Overall, analysis of 167 patients revealed SNP rs12603825 in the *SERPINF1* (also known as *PEDF*) gene remained significantly associated with PDT [185]. Additionally, individuals harboring the AA genotype needed more treatments in a shorter time period compared to those with either the GA or GG genotypes ($p = 0.003$). This remained associated after adjustment for both demographic (age, gender, smoking) and clinical features (baseline PCV lesion size) ($P = 0.0027$). Additionally, SNP rs12603825 also showed worse visual response with overall VA of 0.50 LogMAR for the AA genotype and 0.70 LogMAR for the wild-type GG genotype [185].

Limited studies have been reported investigating the genetic influence on anti-VEGF treatment response in PCV. A Japanese study screened the three SNPs rs1061170 and rs800292 in the *CFH* gene and rs10490924 in the *ARMS2* gene in 56 PCV patients. Analysis of rs1061170 and rs10490924 showed no association with VA or change in VA ($P > 0.05$) after 12 months of ranibizumab treatment. However, rs800292 initially showed association with change in VA after 3 months of treatment ($P = 0.0009$), but this association became weaker after Bonferroni correction ($P = 0.027$) [165]. In addition, all three SNPs were also not associated with clearance of retinal exudative fluid on OCT after 3 and 12 months of ranibizumab treatment [165]. Interestingly, involvement of genetic variants in combination

therapy (PDT and bevacizumab) in PCV has also been reported. A Korean study showed that patients with the TT genotype at SNP rs10490924 in the *ARMS2* gene had poor VA response (0.95 logMAR) compared to patients with either the TG (0.80 logMAR) or GG (0.46 logMAR) genotypes, high fluid leakage on fluorescein angiography (FA) ($P = 0.04$), and less polyp regression ($P = 0.006$) at 12-month follow-up after combination therapy. A similar result was obtained for the SNP rs20011638 in the *HTRA1* gene where individuals with the AA genotype had worse VA (0.94 logMAR) compared to patients with either the AG (0.80 logMAR) or GG (0.40 logMAR) genotypes ($P = 0.02$), fluid leakage on FA ($P = 0.01$), and less polyp regression ($P = 0.002$) [186]. To date, no GWAS study has been published that might unveil novel genetic variants influencing treatment response in PCV.

In summary, all the above mentioned pharmacogenetic studies suggest that known AMD genetic risk variants influence anti-VEGF treatment outcome in AMD patients to some extent and may provide some predictive value in treatment response, but this response is likely small. Similarly treatment response in PCV anti-VEGFs suggests that different genetic variants are likely to influence response to treatment that are different to those exhibited for neovascular AMD. However, in both neovascular AMD and PCV, variable results are present and may reflect ethnic differences, different treatment regimens, outcome time points, number of patients, and differential VA threshold in deciding responders and nonresponders. Other secondary clinical factors such as CMT and fluid clearance also need to be included to assess patients' response to treatment. However, the best pharmacogenetic study should be undertaken in a large number of patients with the same treatment strategy, the same clinical profile parameters, and the use of more advanced high-throughput genotyping technology such as GWAS or whole genome sequencing.

14.7 Conclusion

In conclusion, AMD is a severe blinding disease, and extensive research has been conducted to understand the disease biology. It is not only present in Caucasians but it is also prevalent in Asians. The first ever successful GWAS study identified the *CFH* gene in the complement pathway, and subsequently a number of other genes in the same pathway have led to a convincing role played by the complement pathway in that dysregulation of this pathway plays a role in disease pathogenesis. The effort of many subsequent studies including the AMD Gene Consortium has led to the identification of over 20 genetic loci associated with AMD susceptibility and implicates a number of different biological pathways. In Asia, the GAMA Consortium has reported novel variants in the *CETP* gene associated with neovascular AMD in Asians rather than in Caucasians. This indicates that AMD in Caucasians and Asians share common but also distinct genetic risk variants, reflecting genetic diversity among these two populations.

Currently, anti-VEGF injections are the standard treatment for neovascular AMD, but differential response to known AMD risk-associated genes has so far been reported. Pharmacogenetic studies involving the use of GWAS and exome sequence analysis following treatment response are now being undertaken, and these together with advances in imaging technology will prove extremely useful in dissecting apart which genetic variants are associated with which clinical features of AMD.

PCV presents with different clinical, etiological, and pathophysiological features compared to neovascular AMD which suggests it is a different disease. On the other hand, both PCV and neovascular AMD show similar risk and protective genetic associations suggesting some sharing of common genetic risk factors, and this has led to the view that PCV represents a subtype or a continuum of AMD. Thus, there is continued

debate as to the relationship between PCV and AMD. One reason for this debate is the limited number of large GWAS studies on PCV patients. Such studies may provide opportunities to identify novel genetic variants and hence identify biological pathways that differ from those found in AMD. Additionally, different subtypes of PCV exist and differences in ethnic variation across Asia exacerbate the effective identification of genetic variants. An Asian GWAS or sequencing consortium with a large number of samples from its different countries would play a powerful role in deciphering this issue. Such studies would also have benefit in better understanding the outcome of current treatment modalities which appear to show differences with good short-term outcome VA improvement but longer-term continuous persistence of fluid and less polyp regression.

14.8 Future Aspects

Currently no treatments are available for the GA form of AMD. There are multiple clinical trials currently underway with the most advanced being the phase III clinical trial of lampalizumab (anti-factor D Fab, Roche/Genentech). This trial is also paving the way for personalized treatment outcomes in AMD through utilization of knowledge regarding a specific genetic variant in the complement factor D gene, and thus additional findings in this area will be extremely useful. Recent work undertaken by international AMD consortia, in both Europeans and Asian populations, is also clarifying similarities/differences in both Caucasians and Asians, indicating that there is likely a spectrum of gene variants implicated in disease. The future uptake of next-generation sequencing techniques such as whole exome or whole genome sequencing will hopefully allow for identification of a range of additional common and rare variants in both AMD and PCV. In turn, these should provide a valuable aid in exploring different pathways underpinned by disease-associated genes and help elucidate new biomarkers for detection of disease. In addition, such studies will allow identification of genetic variants responsible for differential response to anti-VEGF treatment in AMD.

Genetic variation explained by SNPs provides one level of variability for AMD and PCV patients; other genetic mechanisms such as through copy number variation (CNVs) and epigenetics will likely lead to further elucidation of both AMD and PCV. Thus despite much intensive research into AMD and PCV, there are still many challenges that remain before we can implement these findings in to the clinic and apply a personalized treatment to every patient.

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Genetics and Pathology of Inflammatory Components on AMD

15

Xiaoxin Li, Lv-Zhen Huang, Peng Zhou, and Chi-Chao Chan

Abstract

Genetic and environmental risk factors of age-related macular degeneration (AMD) are different between Asian and Caucasian. AMD risk factors implicate that inflammation and immunity play a role in the etiology and pathology of AMD. The main pathology of AMD is composed of drusen formation, retinal pigment epithelial cell dysfunction, neovascularization, and photoreceptor cell death. Inflammatory mediators, complement components, chemokines, and cytokines have diverse roles that can recruit inflammatory cells, activate inflammasomes, and promote cell death and/or neovascularization. Exploring the role of genetics, as well as the inflammatory components in AMD onset, progression, and response to treatment, may increase our knowledge of disease pathogenesis and provide novel treatment in the future.

Keywords

Age-related macular degeneration (AMD) • Inflammation • Complement • Inflammasome • Cytokine • Macrophage • Neovascularization • Pathology • Genetics

15.1 Introduction

Age-related macular degeneration (AMD) is the leading cause of central irreversible blindness in the elderly worldwide [1]. There is a higher prevalence of AMD in Europeans than in Asians and no difference in prevalence between Asians and Africans. Due in large to its complexity, the etiology and pathogenesis of AMD remain unclear. Both genetic and environmental factors

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contribute to AMD development and progression. Numerous studies provide evidence that inflammation plays a role in AMD at genetic, histopathological, and proteomic levels. Other known AMD risk factors, such as smoking, hyperglycemia, hyperlipidemia, and oxidative stress, also contribute to inflammation. Here, we briefly discuss the current knowledge on these two topics.

Clinically, AMD can be divided into two forms: a dry (geography atrophy, GA) form, characterized by the appearance of drusen formation and degeneration/atrophy of the RPE (Fig. 15.1a), and a wet form, also called exudative, neovascular, or "wet" AMD, in which abnormal growth of choroidal blood vessels through the Bruch's membrane/RPE that often leads to retinal hemorrhages (Fig. 15.1b). Dry AMD progresses more slowly and manifests with drusen, geographic atrophy of RPE, and photoreceptor dysfunction and degeneration. Drusen are visualized as yellow deposits under the RPE and neurosensory retina. The key feature of wet AMD is the formation of choroidal neovascularization (CNV). Neovascularization occurs in the subretinal space leading to leakage of fluid and blood, hemorrhage, RPE detachment, and scarring. The initial symptoms of AMD include loss of central visual acuity, a subjective impression of the curvature of straight lines or metamorphopsia, and a gradually enlarging central scotoma.

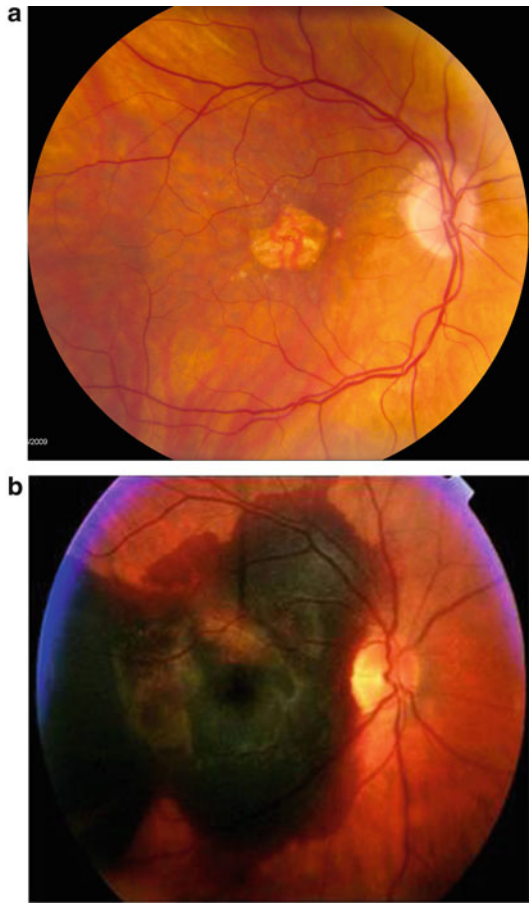

Fig. 15.1 Clinical photographs of AMD. (**a**) An AMD maculae with geographic atrophy; (**b**) an AMD maculae with exudative/neovascular lesion and explosive hemorrhages

15.2 Genetic Aspects: Asian Perspective

AMD is a multifactorial disease with multiple environmental and genetic components. Although, in some cases, AMD appears to run in families, it usually does not have a clear-cut pattern of inheritance. Many epidemiological studies have shown differences in the prevalence based on ethnicity with prevalence in Caucasians than Asians and Africans. Such ethnic differences may reflect genetic as well as environmental factors. Frequency and subtypes of late AMD are different between Asians and whites. Polypoidal choroidal vasculopathy (PCV) is another important form of maculopathy found in a significant proportion of elderly Chinese and Japanese patients, and it manifests as orange-reddish nodules or polyp-like structures at the posterior pole [2]. It is reported that early and late AMD in Asian populations aged 40–79 years overall were 6.8% and 0.56% [3]. When stratified by gender, Asian men had higher point estimate of late AMD prevalence (18.6%) compared with white men (10.1%), while Asian women had lower point estimate prevalence (2.6%) than white women. PCV lesions are frequently observed in Asian patients with exudative AMD [4, 5]. In hospital-based

studies in Asia, the frequencies of PCV lesions among patients with exudative AMD were 23–54.7% in Japanese and 22.3–33.5% in Chinese [3–5]. The dominance of neovascular AMD cases over geographic atrophy cases in Asians is consistent with a higher frequency of PCV cases in Asians with neovascular AMD [3]. To clearly understand the epidemiology of late AMD subtypes and the influences of possible variants such as PCV, future studies are needed that include specific examinations such as fluorescein and indocyanine green angiography, or at least optical coherence tomography, to classify AMD subtypes and identify the presence of PCV in older Asian populations.

The best-studied genes associated with AMD are involved in complement system, particularly complement factor H (CFH), C2/CFB, and C3 [6]. Complement system consists of over 40 proteins and cell-surface receptors and can be activated through three pathways: the classical, alternative, and mannose-binding lectin pathways which all lead to the production of C3 and C5 convertase enzyme complexes. The enzyme complexes then cleave C3 into the anaphylatoxin C3a and the opsonin C3b and C5 into the anaphylatoxin C5a and into C5b, respectively.

CFH is a member of the regulators of complement activation family and is a complement control protein. Its principal function is to regulate the alternative pathway of the complement system, ensuring that the complement system is directed toward pathogens or other dangerous material and does not damage host tissue. CFH is expressed at the RPE-choroid level and is increased in AMD lesions. CFH is one of the first reported genes that are associated with AMD [7]. Individuals with a CFH variant that substitutes a tyrosine amino acid for a histidine at position 402 (rs1061170 (Y402H)) increased the likelihood of developing AMD 4.6-fold if present on one allele and 7.4-fold if present on both alleles [7]. Further CFH studies found that the CFH haplotype significantly increased the risk for AMD with odds ratios between 2.45 and 5.57 and that a common variant likely explains approximately 43% of AMD in older adults [8–13].

CFH risk allele rs1061170 is present in 35% of Caucasian populations and was estimated to play a role in almost 60% of AMD at the population level [14]. However, the frequencies are low in Chinese (2.9 and 8% in control population and 5.8 and 10% in AMD from reports in Hong Kong and Beijing, China, respectively) [15, 16] and Japanese (4% both in control and AMD) [17]. No association between re1061170 in CFH and AMD was also replicated in another Japanese study [18]. In a case-control study of 344 neovascular AMD and 368 PCV Chinese patients, rs1061170 and 10 other CFH SNPs were significantly associated with both neovascular AMD and PCV in Chinese [19]; however, rs1065489 was not associated with AMD that was reported in Caucasian patients [19, 20].

Association studies have also demonstrated between AMD and other different complement genes. Variations in the C3 gene are associated with AMD. C3 plays a central role in complement system and contributes to innate immunity. Its activation is required for both classical and alternative complement activation pathways. A nonsynonymous C3 variation Arg80Gly (rs2230199) is associated with AMD [21–23]. Another C3 variant (rs1047286) is also associated with AMD [8, 22]. Furthermore, some rare variants, such as p.Arg1210Cys and p.Pro314Leu, are associated with high risk of AMD [24, 25]. CFI is a serine protease that cleaves and inactivates C4b and C3b. CFI regulates activation of alternative pathway. A C>T transition (rs10033900) located downstream the 3′UTR of CFI gene has been shown to be associated with AMD [26, 27]. Recently, some rare variants in CFI, such as p.Gly119Arg and p.Pro50Ala, are associated with high risk of advanced AMD [28, 29]. Individuals with AMD and rare CFI variants commonly have low serum FI antigenic levels, which support the role of impaired FI-mediated regulation of the complement system's alternative pathway in the pathogenesis of AMD [30]. Genetic association is also reported between CFD (rs3826945) and AMD in a small discovery case-control series and replicated in a combined cohort meta-analysis

of 4765 cases and 2693 controls [31]. In this study, there is a significant increase in plasma CFD levels in AMD cases compared with controls, which is consistent with a role for CFD in AMD pathogenesis.

C2 and CFB are described together because of similarities in structure, function, and genetic characteristics [32]. The C2 gene, located on 6p21.33, encodes a serum glycoprotein that functions as part of the classical complement pathway, which is involved in innate immunity and inflammation [33]. Two polymorphisms, rs9332739 G > C and rs547154 G > T, have been implicated in AMD. The C2 polymorphisms may be associated directly with AMD or indirectly through the high level of linkage disequilibrium that exists between C2 and CFB, which is located downstream on the same chromosome [8, 34, 35]. The protective effect of C2/CFB variations may be due to a reduction in formation of either classical or alternative pathway C3 convertase. Montes et al. found the CFB 32Q variant (rs4151667) had up to four times less C3b binding affinity than the non-risk 32R protein, with consequent reduction in C3 convertase formation [36].

Understanding the links between the genetic susceptibility data and the clinical symptoms should provide a framework for a deeper understanding of AMD pathogenesis and consequently contribute to identifying new therapeutic targets to slow or halt vision loss associated with the disease.

15.3 Pathology of Inflammation in AMD

Numerous studies including the association of candidate immune genes and AMD have provided evidence that inflammation has a role in AMD pathogenesis including known AMD risk factors, such as smoking, hyperglycemia, hyperlipidemia, and sequence variants associated with disease susceptibility of immune-regulated genes. Mounting evidence from clinical and laboratory-based studies from genetic, proteomic, histopathological, and pathological aspects appointed a great role of immune system in the pathogenesis of AMD. Following a series of independent research papers in late 2005 suggesting a link between the body's immune system and AMD [6], further investigations established the alternative complement system as a potentially critical player that may help scientists to join the dots between drusen, a small accumulation of extracellular material between Bruch's membrane and retinal pigment epithelium (RPE), and the symptomatic degeneration of the macula [37, 38].

Drusen, which accumulate in Bruch's membrane between the retinal pigment epithelium (RPE) and the underlying choroid, are the major pathological hallmark common to both dry and wet AMD (Fig. 15.2 a–c) [39, 40]. Proteomic studies of human drusen which were performed by immunohistochemistry, liquid chromatography-tandem mass spectrometry (LC-MS/MS), or isobaric tag for relative and absolute quantitation (iTRAQ) technology identified many immune response and host defense proteins, including complement and complement-associated proteins such as C3, C5, C5a, C9, complement factor B, complement factor H (CFH), and clusterin [41]. Amyloid-beta (Aβ), a drusen component found in AMD eyes, is also a major proinflammatory factor that has been extensively studied in the context of AMD. Kurji et al. discovered that inflammation-associated genes and immune response pathways were the predominant responses of RPE to oligomeric Aβ stimulation [42]. Using intravitreal injections of Aβ in rodents, the RPE demonstrated elevated levels of NLRP3 and IL-1β in rodents [43]. Drusen may lead to CNV by reducing RPE adhesion to Bruch's membrane and allowing for vessels to enter the sub-RPE space. Also, inflammatory cells found in the subretinal space near drusen secrete angiogenic factors, stimulating blood vessel growth as part of the wound healing response [44]. We speculate that the development of AMD depends on the type of immune response when drusen accumulate. In the neovascular form of AMD,

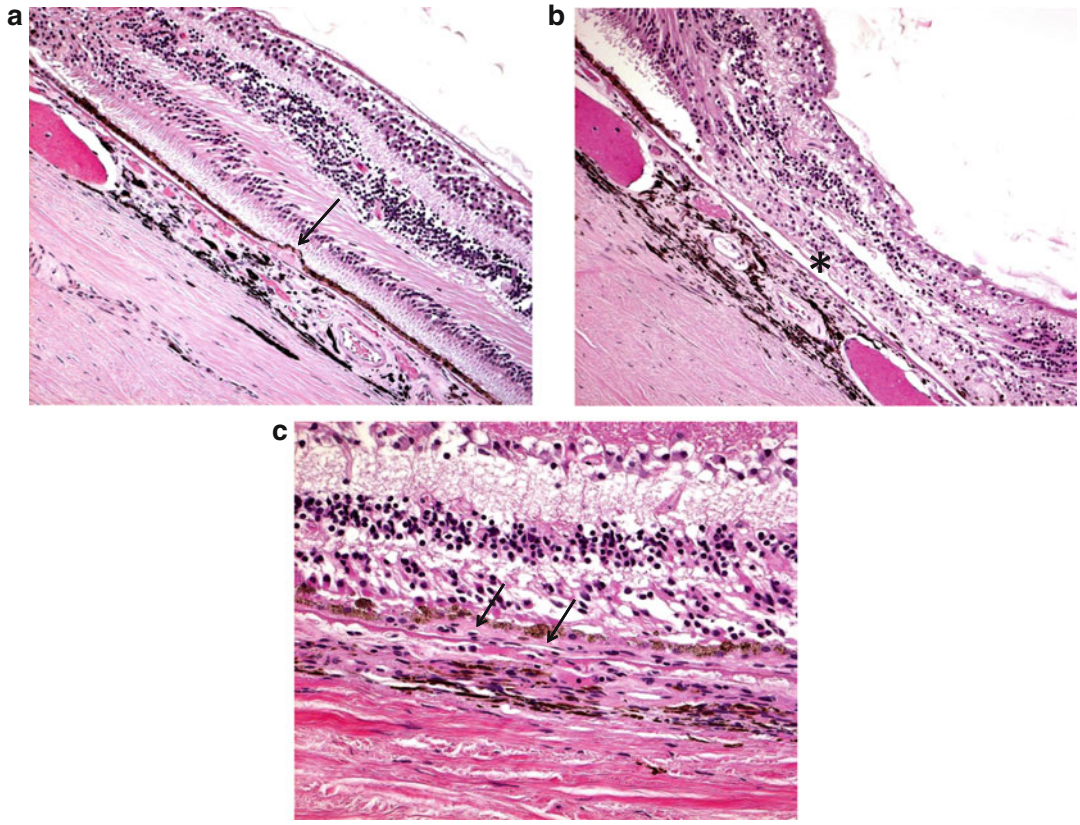

Fig. 15.2 Microphotographs of AMD lesions. (**a**) A single druse (*arrow*) in the Bruch's membrane beneath RPE; (**b**) a typical geographic atrophic lesion with loss of photoreceptor and RPE cells (*asterisk*), irregular Bruch's membrane, and disorganized outer plexiform and inner nuclear layers; (**c**) a thin layer of neovascular fibrous tissue with small vascular lumens (*arrows*) between the degenerated RPC and Bruch's membrane (hematoxylin and eosin, original magnification, x100)

breakdown of the blood–retinal barrier provides circulating immune cells with unusual access to a highly immunogenic environment, which results in inflammatory cell recruitment and activation, together with local inflammatory cells and cytokines; they initiate a VEGF-A-dependent or VEGF-A-independent neovascular response to produce results in a leaky, abnormal vascular network. In the case of geographic atrophy, degenerated RPE and photoreceptors damage neighboring cells by releasing toxic inflammatory stimulators and cytokines [45]. Overall, although underlying etiology of AMD remains unknown, there is clear evidence that dysfunction in the photoreceptor layer, RPE, underlying extracellular matrix, and choriocapillaris is a significant factor in the development and progression of the disease.

Central to AMD pathogenesis, the RPE undergoes significant changes in structure and function that predispose individuals to disease processes associated with AMD. Deciphering the nature of RPE cell death as well as photoreceptor degeneration and death in AMD will not only be crucial to understanding AMD pathogenesis but also instrumental for designing targeted therapeutic agents for AMD. Different cell death pathways are involved in the retina of AMD eyes [46]. Apoptosis is a tightly regulated process of programmed cell death, which is crucial for development and homeostasis. Morphologically, apoptosis is characterized by cytoplasm shrinkage, chromatin condensation, nuclear

fragmentation, formation of apoptotic bodies, and maintenance of the plasma membrane. It involves caspase activation, mitochondrial outer membrane permeabilization, DNA fragmentation, and lysosomal membrane permeabilization [47]. Depending on cell type and insult, apoptosis can be executed by different pathways. The extrinsic pathway involves cellular membrane receptors, and intrinsic pathway is mediated through mitochondria [48]. Dunaied et al. analyzed dying cells in GA retinas and found TUNEL-positive RPE and photoreceptor cells at edges of atrophic areas, suggesting apoptotic RPE cells in those AMD patients [49].

Necrosis/necroptosis is used to be considered a passive type of cell death induced by physical, chemical, and/or biological insult, and it can induce an inflammatory and immune response. The fundamental features of necrosis include cellular energy depletion, damage to membrane lipids, and loss of function of homeostatic on pumps/channels. Major morphological changes that occur in necrosis include cell swelling; formation of cytoplasmic vacuoles; distended endoplasmic reticulum; formation of cytoplasmic blebs; condensed, swollen, or ruptured mitochondria; disaggregation and detachment of ribosomes; disrupted organelle membranes; swollen and ruptured lysosomes; and eventually disruption of the cell membrane [47]. Previous research indicates that RPE cell necrosis on response to oxidative stress has a detrimental role in inflammation [50]. Necrotic features such as cell swelling, rounding, and vacuolization have been observed in in vitro models of oxidative stress-induced RPE death, AMD mouse models, and AMD (especially dry AMD) patients [47].

Autophagy involves the formation of a double-membrane structure (autophagosome) that engulfs cytoplasmic proteins, lipids, and damaged organelles and contributes to the equilibrium between the production of protein and organelles and their clearance (Fig. 15.2). Autophagy process can be divided into induction, initiation/nucleation, elongation and closure, maturation and fusion, and, finally, degradation steps [51]. This process is mediated by more than 30 autophagy-related (Atg) proteins. Some exosome and autophagy markers can be detected in drusen [52]. In addition, autophagy plays an important role in the regulation of inflammatory responses especially the release of IL-1 family cytokines. In addition, autophagy can influence IL-1β [52]. Autophagy is closely linked to inflammasome or by directly limiting IL-1β availability. Autophagy has also been shown to control IL-1β secretion by targeting pro-IL-1β for lysosomal degradation and by regulating NLRP3 inflammasome activation. Stimulating autophagy in vivo decreased serum levels of IL-1β in response to LPS challenge [53]. In normal circumstances, autophagy controls the activation of the NLRP3 inflammasome, for example, by degrading the inflammasome components and effector molecules. Conversely, when autophagy declines, inflammasomes become activated—most probably through the dysregulation of mitochondrial homeostasis [53–55]. Autophagy could function as a mechanism to limit excessive inflammation by directly eliminating active inflammasome complexes [54]. Ardeljan et al. reported that IL-17 is aberrantly expressed in the AMD macular lesions compared to non-diseased controls. If ARPE-19 cells are treated with IL-17A, various pathological ultrastructures that are consistent with changes observed in RPE of AMD lesions can be found including autophagosomes (Fig. 15.2), mitochondrial damage (Fig. 15.2), and nuclear psychosis and necrosis [46]. Due to proapoptotic protein expression and decreases in cell viability, a large number of autophagosomes could be detected in multipotent retinal stem cells (RSCs) by IL-1β-induced VEGF levels, which may have important implications in CNV development associated with wet AMD [56]. Considering the above results, one might expect autophagy induction to be a reasonable treatment for early macular degeneration, when signs of RPE damage are just beginning. On the other hand, if the RPE is damaged past a critical point, such as in the later stages of AMD, autophagy might cause cell death and thereby exacerbate the disease. So for autophagy, timing is of the essence [57].

Pyroptosis, a mode of programmed cell death, characterized by formation of membrane pores and osmotic lysis (Fig. 15.3), differs from apoptosis, is derived from the Greek "pyro" (fire) to denote the release of proinflammatory mediators, and is dependent on the activation of caspase-1 and the inflammasome [58, 59]. DNA cleavage and nuclear condensation can occur during pyroptosis, which are distinct from DNA laddering seen in apoptosis since nuclear integrity is preserved [60]. Pyroptosis involves the secretion of inflammatory cytokines including IL-1β and IL-18. Doyle et al. observed that overexpression of pro-IL-1β and pro-IL-18 caused cell swelling, which might be an indication of pyroptosis [61]. Our knowledge now of the amount of inflammation that occurs within AMD's phenotypes, as well as the involvement of the NLRP3 inflammasome, suggests possible pyroptosis in addition to apoptosis as a death pathway [38].

15.3.1 Complement Factors

Recent research found that CFH is an innate defense protein against oxidative stress [62]. Results from animal models lacking immunoglobulins showed that over 55% of peptides bound to MDA could be attributed to CFH. Mapping of the binding site for MDA on CFH showed that it crossed the amino acid position 402, highlighted in the original genetic association studies, and, most importantly, the H402 variant of CFH showed reduced MDA binding by up to 23% in the plasma of heterozygotes and up to 52% in homozygotes. Normal CFH then appears to protect against inflammation by inhibiting the complement pathway; however, once mutated, the ability for CFH to control the inflammation associated with AMD appears to be lost. Functioning CFH is able to suppress the inflammatory response by mopping up the MDA adducts [62]. Moreover, CFH plays an important role in the regulation of the alternative pathway. CFH binds C3b and accelerates the decay of the alternative C3 convertase (C3bBb). It also acts as a cofactor for the inactivation of C3b by complement factor I (CFI) [32, 63]. CFH binds to cell surfaces to regulate amplification of the alternative complement pathway resulting from spontaneous C3b deposition, which occurs on any surface in contact with blood [32, 63].

C3 is the most abundant component of the complement pathway and may play an important role in the pathogenesis of AMD. *C3* mRNA can be detected in the neural retina, choroids, and retinal pigment epithelium. Local C3a levels increase in experimental model of wet AMD [64]. Eliminating the C3 gene protects from CNV development after exposure to laser in C3 gene knockout mice [65]. Furthermore, C3a may increase VEGF secretion and recruitment of monocytes via ICAM-1 production [65].

Human CFI deficiency leads to low plasma C3 and may cause recurrent bacterial infections in children. The effect of CFI variations on AMD formation may be due to a reduction in the degradation of C3b. A recent study found that plasma and sera from cases carrying the p. Gly119Arg substitution mediated the degradation of C3b to a lesser extent than those from controls [28]. CFD is involved in alternative complement pathway, where it cleaves factor B. CFD is a serine protease that stimulates glucose transport for triglyceride accumulation in the adipose cells and inhibits lipolysis. Human CFB is a protein encoded by the *CFB* gene, a component of alternative pathway of complement activation. The polyadenylation site of this gene is near the 5′ end of the C2 gene, which involves in classical pathway of complement activation. A protective effect of alleles in C2 and CFB is reported, although it may differ across ethnicities.

Collectively, human and animal studies discussed above provide strong evidence that complement activation is an important element in the development of AMD. A cyclic peptide, POT-4, binding to C3 to inhibit its activation, is the first complement inhibitor for AMD that entered into a phase I trial [66]. Lampalizumab, a humanized Fab fragment of the murine anti-CFD mAb 166-32, has shown positive effects on dry AMD in phase I and II (MAHALO, NCT 01602120) studies. Two new phase III clinical

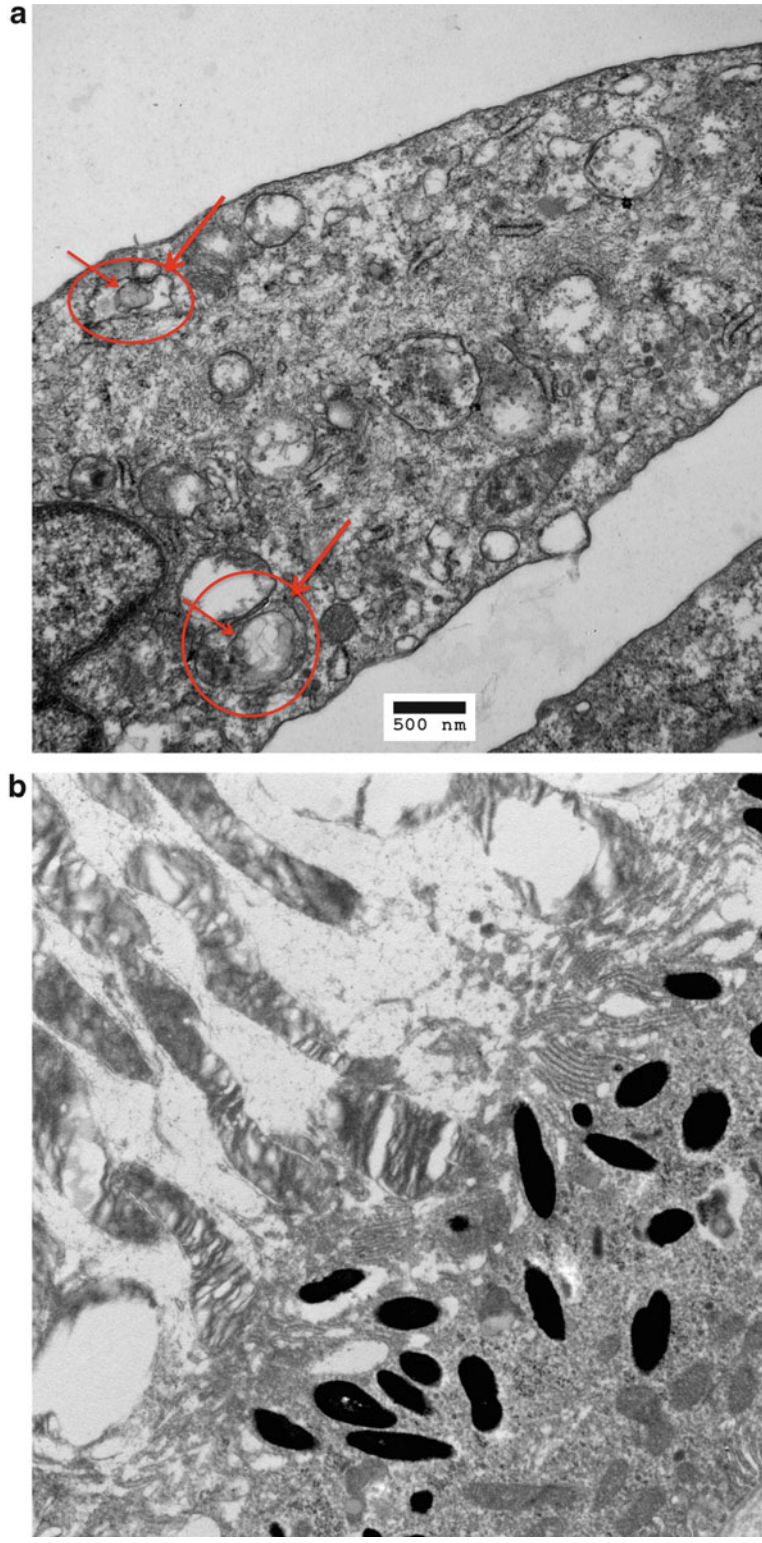

Fig. 15.3 Transmission electron microphotographs of AMD cells. (**a**) Autophagosomes (*circles*) surrounding with double membrane (*arrows*); (**b**) vacuolated cytoplasm of the degenerated photoreceptors

studies of CHROMA (NCT 02247531) and SPECTRI (NCT 0247479) are currently underway [67]. The limited complement-based therapy is progressed to phase III clinical trial that highlights our lack of basic understanding about the mechanisms by which complement factors influence AMD development.

15.3.2 NLRP3 Inflammasome and Cytokines

Activation of extracellular signal-regulated kinase 1 (ERK1) and ERK2 downstream of C3aR ligation leads to increased efflux of ATP to engage its cell-surface receptor P2X purinoreceptor 7 to activate the NOD-, LRR-, and pyrin domain-containing protein 3 (NLRP3; also known as NALP3) inflammasome [68]. C5aR1-mediated signals also contribute to inflammasome activation [69]. Activated complement components as well as complement regulators are present in drusen [70]. Complement component C1q, an AMD biomarker found in drusen, has been reported to activate the inflammasome; on the other hand, that drusen themselves can also cause NLRP3 activation and secretion of IL-1β and IL-18 in peripheral blood and macular cells of both dry and wet AMD eyes in spite of specific molecular mechanism remains to be defined [71–73].

The inflammasomes are intracellular, multiprotein complexes whose molecular composition is stimulus dependent. NLRP3 is the best-studied inflammasome owing to its involvement in immunity to a multitude of bacterial, viral, and fungal pathogens and to its expanding roles in inflammation and diseases. NLRP3 inflammasome is a protein complex that is comprised of a NOD-like receptor (NLR) protein, the pro-protein, procaspase-1, and adaptor molecule ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain). It appears as a large multimetric danger-sensing platform, response to a wide range of molecules from endogenous such as ATP and amyloid-β, or exogenous danger signals such as bacterial toxins and microbes; promotes the cysteine protease, caspase-1; and mediates the cleavage of inactive pro-IL-1β and IL-18 into their mature proinflammatory forms [74–77]. An increase in vascular endothelial growth factor A (VEGF-A), associated with neovascularization, also resulted in NLRP3 inflammasome activation in RPE cells in a transgenic mouse model of AMD, mediated by oxidative damage. Interestingly, NLRP3 deficiency in this mouse model reduced the number of VEGF-A-induced CNV lesions and RPE barrier breakdown, suggesting involvement of the inflammasome in the pathology of both forms of AMD [78].

Tarallo et al. found that NLRP3, IL-18, and caspase-1 abundance was significantly elevated in the RPE of human eyes with dry AMD compared to normal age-matched control eyes [71]. Tseng et al. showed NLRP3 expression at sites of geographic atrophic or neovascular lesions in AMD eyes but not in the RPE of lesion-free areas of the retina [72]. Ambati et al. found increased levels of IL-1βmRNA in the RPE of individuals with dry AMD [45]. Doyle and colleagues reported that mice immunized with carboxyethylpyrrole (CEP), an oxidative protein modification derived from docosahexaenoate-containing lipid, developed AMD-like lesions, drusen accumulation, and other characteristics of dry AMD and NLRP3 and caspase-1 were induced near AMD-like lesions in these mice [79]. They also demonstrated that deletion of NLRP3 or IL-18 exacerbated CNV formation in the laser-induced CNV mouse model [79, 80].

In an in vitro study using adult human RPE cell lines of ARPE-19 and another cultured primary human RPE cell (hRPE), NLRP3, IL-1β, and IL-18 expression and caspase-1 cleavage were significantly enhanced by inflammatory or oxidative stress which were mimicked by TNF-α, LPS+TCDD, or H_2O_2 stimulation [81]. On the other hand, knockdown of NLRP3 expression in ARPE-19 showed less caspase-1 cleavage as well as reduced IL-1β and IL-18 expression in response to stimulation with TNF-α. Compared with controls, the presence of pyroptosis, autophagosomes, cytoplasmic vesicles, mitochondrial damage, cytoskeleton disruption,

glycogen and lipid droplet accumulation, and nuclear membrane separation in IL-1β- and IL-18-stimulated mouse primary RPE and mouse retinal stem cells (RSCs) was noted [82].

There is evidence that IL-1 can synergize with IL-23 to induce secretion of IL-17 from murine T cells or promote induction and activation of human Th17 cells [81, 83, 84]. In addition, IL-1 can induce IL-6 production that stimulates the differentiation of naïve T cells in Th17 cells [85]. The IL-17 cytokine family includes six members named A–F. IL-17 RC serves as an essential subunit of the IL-17 receptor complex that mediates the signal transduction and proinflammatory activities of IL-17A and IL-17F.

Higher levels of alternative complement activation such as CFB, C3a and C5a, and Th17 cytokines were detected in the blood from AMD cohort [86, 87]. Serum levels of IL-17 and IL-22, a Th17 family cytokine, were significantly higher in AMD patients compared to age-matched non-AMD individuals [88]. In addition, C5a anaphylatoxin can promote Th17 cytokine expression from human CD4+ T cells. IL-17RC, a member of IL-17R family and the primary receptor for IL-17A, mediates the signal transduction and proinflammatory activities of IL-17A and IL-17F. Hypomethylation of the IL-17RC promoter was associated with AMD [89]. The epigenetic alteration may lead to elevation of IL-17RC transcript and protein in peripheral blood as well as in macular cells of dry or wet AMD patients than in non-AMD controls. Since the IL-17RC subunit plays a key role in modulating the IL-17 response [90], the association of IL-17RC with AMD suggests that IL-17 could be an important player in AMD pathogenesis.

Humanized anti-IL-17 antibodies, secukinumab and ixekizumab, are now in phase II trial for the treatment of rheumatoid arthritis [91]. Recently, two additional small-molecule inhibitors that reduce NLRP3 activation have been reported. The ketone body, hydroxybutyrate (BHB), which serves as an alternative source of ATP during energy-deficit status, was found to specifically inhibit a variety of stimuli triggering NLRP3 inflammasome activation. In animal models of NLRP3-mediated diseases such as Muckle–Wells syndrome, BHB-complexed nanolipogels and a ketogenic diet strikingly attenuated caspase-1 activation and IL-1β secretion. MCC950, a highly selective inhibitor of the NLRP3 inflammasome, has been shown to reduce IL-1β production in other disease models, providing significant strides toward therapeutic application [92]. A phase 1 safety trial for a neutralizing monoclonal antibody to IL-18 (GSK A18110040) has been carried out but has not been well reported to date [93]. Considering the role of these chemokine receptors during the course of AMD, targeting any of these factors may prove beneficial for patients.

15.3.3 Ubiquitin–Proteasome System (UPS)

Ubiquitin is a small regulatory protein that has been identified in almost all tissues of eukaryotic organisms. Ubiquitylation is the posttranslational modification process by which ubiquitin is attached via an isopeptide bond to lysine residues on a protein. Ubiquitin affects on proteasome- or lysosome-mediated protein degradation, cell signal transduction, and protein activity regulation. Mounting evidence has implicated the involvement of ubiquitylation in AMD pathophysiology. Recently, Huang et al. screened the susceptibility loci for neovascular AMD from East Asian cohorts in the coding exome using whole-exome sequencing technology and identified a nonsynonymous amino acid substitution, rs7739323 (encoding for a V4M at position 379 in UBE3D, ubiquitin protein ligase E3D) [94]. They detected a significant association between rs7739323 and AMD risk. UBE3D, also known as ubiquitin-conjugating enzyme E2C-binding protein (UBE2CBP), accepts ubiquitin from specific E2 ubiquitin-conjugating enzymes and transfers it to substrates, generally promoting their degradation by the proteasome. Moreover, ablation of the UBE3D protein leads to retinal structural changes and abnormal electroretinograph (ERG) in $UBE3D^{+/-}$ heterozygous mice. Electron microscopy showed more loose retinal pigment epithelium microvilli and more

pigment granules in 3-month-old UBE3D$^{+/-}$ heterozygous mice than in wild-type mice. Therefore, this study demonstrates a potential link between AMD and the UPS [94]. Rodgers et al. found that linear ubiquitin assembly complex (LUBAC) is also essential for NLRP3 inflammasome activation, independent of NF-κB activation [95].

15.3.4 Inflammatory Cells: Macrophages, γδ T Cells, and Microglia

The development of AMD is accompanied by the recruitment of inflammatory cells, particularly macrophages [96]. Macrophages, initiating the production of inflammatory cytokines and chemokines and complement factors including vascular endothelial growth factor, are the most prominent inflammatory cells observed in AMD tissue [97]. Macrophage activation is highly relevant to AMD pathology, as identified in regions of RPE atrophy, breakdown of Bruch's membrane, and choroidal neovascularization [98]. Macrophages and dendritic cells (resident macrophages) are not normally present in the retina but reside in the underlying choroid. In cases of breakdown of the blood–retinal barrier, these cells are recruited from the underlying choroid or from the systemic circulation into the retina where they modulate disease [45]. Numerous studies have identified the presence of macrophages in or near AMD lesions, especially in the drusen, Bruch's membrane, choroid, and retina [99, 100]. Macrophages can polarize to at least two functional phenotypes, M1 and M2, with markedly different expression of receptors, chemokines, and cytokines [101]. The M1 phenotype that is triggered by TNF-α or LPS to produce inflammatory cytokines and highly toxic reactive oxygen and nitrogen species has a proinflammatory and anti-angiogenic effect; M2 phenotype that is activated by IL-4 and IL-10 and produces anti-inflammatory and pro-angiogenic factors has a pro-angiogenic and anti-inflammatory effect [38, 102]. Depending on the environment, macrophages can be classically (M1) or alternatively (M2) activated; they can easily be converted and exchanged their phenotypes. Preliminary evidence implicates that RPE cells can induce chemotaxis of monocytic cells [103]. Monocytes can be recruited and primarily mediated by the chemokine receptors CCR2 and CX3CR1, and their ligands CCL2 (monocyte chemoattractant protein-1, MCP-1) and CX3CL1 (fractalkine), and differentiate to macrophages that may affect the course of any stages of AMD [104–107]. An aberrant polarization and functional phenotype were observed in the macular lesions of AMD eyes [108].

γδ T cells, rapid and potent producers of cytokines, are an unconventional T-cell subset [109]. IL-18 and IL-23 bind to their respective receptors on Th17 and γδ T cells to induce production of IL-17A, IL-17F, GM-CSF, and IL-22. γδ T cell itself can also express a variety of chemokine receptors and cytokine receptors to induce IL-17 [81]. Thus, activation of the inflammasome, consequently processing the cytokines IL-1 and IL-18, implicates an important role in the generation of IL-17-secreting γδ T cells [110].

Microglia is another immune cell type that may involve in AMD. Morphological studies with AMD patients' retinas revealed activated microglia in the photoreceptor layer and subretinal space that incorporated rhodopsin-positive particles, indicating high phagocytic activity [111]. Isolated microglial cells from 18-month-old mice showed morphological signs of activation and expressed proinflammatory markers, indicating low-grade immune activation in the aging retina [98]. Halle et al. reported the activation of NLRP3 inflammasome by lysosomal destabilization which increased the release of IL-1β in murine microglia [112].

Chronic inflammation can be a causative factor for the development of AMD, which results in endothelial dysfunction, facilitates the interactions between modified lipoproteins, monocyte-derived macrophages, T-cells and normal cellular elements of the retinal vascular wall. Activated macrophages and microglia may secrete chemokines and cytokines, causing further cellular damage, Bruch's membrane

degradation, and angiogenesis [113–115]. There is a wealth of evidence implicating inflammatory components in key processes during angiogenesis that include endothelial cell proliferation, migration, and deregulated blood vessel growth [116]. Taken together, all these data provide strong evidence of inflammatory component activation in AMD pathogenesis.

15.4 Summary

Genetic and molecular studies have provided convincing evidence that inflammatory components are potentially involved in AMD of Asians. Future efforts should attempt to improve the detailed inflammatory pathways in AMD in order to discover predictive biomarkers as well as disease prevention and therapeutic targets.

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Genome-Wide Association Study of Age-Related Eye Diseases in Chinese Population

16

Li Jia Chen, Shi Song Rong, and Chi Pui Pang

Abstract

Since the first successful genome-wide association study (GWAS) reported in 2005 on mapping a disease gene – complement factor H (*CFH*) – for age-related macular degeneration (AMD), a large number of genetic loci for eye diseases have been identified by GWAS. The subsequent GWAS that again successfully identified an AMD gene on the *HTRA1/ARMS2* locus happened to be started in Chinese study subjects. Technological advancements and availability of large sample sizes have contributed to gene mapping for many eye diseases. GWAS, with Chinese patients as the initialization study cohort, has detected new disease genes of complex eye diseases, including AMD, primary open-angle glaucoma (POAG), primary angle-closure glaucoma (PACG), myopia, diabetic retinopathy, and Vogt-Koyanagi-Harada syndrome. Multiple quantitative traits and endophenotypes, such as central corneal thickness, corneal curvature, astigmatism, anterior chamber depth, axial length, and intraocular pressure, have also been investigated by GWAS for gene identification. Results of replications and validations with cohorts of other ethnicities are consistent in association in some studies and different in statistical significance in others. Ethnic-specific sequence alterations are known in some candidate genes. Both similarities and differences exist in the patterns of sequence alterations and phenotype-genotype associations as reported in Chinese when compared with other ethnic groups, even among oriental populations. With the advent of GWAS and other high-throughput sequencing technologies, increasingly detailed genome information is obtained. There are increasing numbers of reported GWAS with Chinese as initial cohorts. With the collection of large amount of genome information, downstream gene function analysis and epigenetic studies are in process with a view to

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throw light on disease mechanisms through understanding the functions and properties of eye disease genes.

Keywords
GWAS • Eye disease genes • Gene mapping • SNP • Chinese

16.1 Introduction

Genome-wide association studies (GWAS), utilizing high-throughput genotyping technologies and fast data processing logarithms, have led to identification of hundreds of gene loci for over 10,000 phenotypes during a relative short span of 10 years. The success of GWAS is spectacular to map genes for complex diseases of overlapping phenotypic features and complex genetic architecture. Even disease entities with late disease onset and consequently rarity of big pedigrees, rendering conventional linkage analyses difficult, have gene loci convincingly mapped by GWAS. The first two successful GWAS to map disease genes in ophthalmology are on age-related macular degeneration (AMD) [1, 2], a late-onset and common disease with considerable clinical heterogeneities. It is notable that the both GWAS utilized primary study cohorts of small size. Genome screening of 96 patients and 50 controls in a Caucasian cohort, involving 116,204 single-nucleotide polymorphisms (SNP), identified a missense variant, p.Tyr402His (rs10611170), in the complement factor H (*CFH*) gene to be strongly associated with the disease [1]. This discovery has given proof to a role of the inflammatory and complement system in the pathogenesis of AMD, since CFH is a key regulator of the complement system of innate immunity. As for exudative AMD, genome-wide genotyping of 97,824 SNPs in a Chinese cohort of 96 AMD patients with stage 5 exudative AMD and 130 - age-matched healthy controls identified a SNP rs11200638 in the 10q26 region [2]. It is located at the promoter region of *HTRA1*, serine peptidase 1 in the (high-temperature requirement) serine protease family.

The primary study cohorts of these two early successful GWAS are notably small, largely attributed to the outstandingly strong association of the identified gene variants with the disease. Genome screening of complex diseases yields to large sample sizes to attain sufficient statistical power for detection of specific gene variants for diseases. Apart from SNP-trait associations requiring $P < 10^{-8}$, magnitude of the minor allele frequency (MAF) is another weighting effect in power calculation and estimation of sample size requirements. Most GWAS involve more than 10,000 study subjects even at the discovery stage. In addition, meta-analysis of data obtained from other cohorts contributes greatly to affirmation of genetic associations. Again, many thousands of study subjects are involved.

Ethnicity is another factor affecting SNP-trait association. For example, the p.Tyr402His variant in *CFH*, so strongly linked to AMD in Caucasians, is rare in Chinese. Detailed genotyping analysis in Chinese revealed rs800292, attributing to p.Ile62Val in exon 2, is associated with Chinese AMD ($P = 0.00008$) for allele association, but not rs1061170 (p.Tyr402His, $P = 0.2$) [3]. Remarkable ethnic differences exist in genetic architecture. In Caucasians, rs3753394 and rs800292 are in a same linkage disequilibrium (LD) block, while rs1061147, rs1061170, rs380390, and rs1329428 are in another LD block. However, in Chinese, the whole *CFH* gene is in the same LD block, containing rs1061147, rs1061170, and rs380390 not in LD with flanking regions [3, 4]. Further studies showed no interaction of smoking with *CFH* and *HTRA1* to AMD risk. It is also noted that there is joint action of *CFH* and *HTRA1* as independent multiplicative effect without significant interaction [5].

Fine gene mapping requires segregation of ethnicities to enhance unequivocal detection of disease genes. The monumental work of the AMD Gene Consortium has led to identification of 19 loci for AMD involving more than 17,100 advanced AMD patients and more than 60,000 healthy controls largely from Caucasians [6]. Genetically, some ethnic groups may be "closer" to each other. The consortium of Genetics of AMD in Asians (GAMA) was organized in 2012 to group Korean, Japanese and Chinese for AMD genetics with primary cohorts recruited in Seoul, Kyoto, Singapore and Hong Kong. So far two studies utilizing whole-exome sequencing have been reported [7, 8]. In 2014, the Asian Eye Genetics Consortium (AEGC) was established to encourage and coordinate eye genetic studies on Mendelian and complex diseases among all Asian populations.

Primary Chinese cohorts on complex eye diseases, notably glaucoma, AMD and myopia have been organized for GWAS, with replication studies involving other ethnic populations. Some studies attended to traits and endophenotypes. There are also successful GWAS involving study cohorts only of ethnic Chinese. Large amount of genetic information has been collected and will lead to further genomic and gene studies. New gene information is also expected to be unraveled.

16.2 Genetic Epidemiology

Both environment and genetics affect the occurrences of diseases, especially complex diseases, in different populations of various geographic locations. In eye diseases, myopia is an example of particular higher occurrence in oriental populations of Japanese, Korean and Chinese than in Indians and Caucasians even among people of similar education background and socioeconomic conditions. Lifestyles certainly play a role. Reading habits, near work, and outdoor activities are proven contributing factors. Constitutive genetic factors are also evidenced from parental background, twin studies, and segregation analyses [9]. With identification of myopia genes, ethnic differences in allelic frequencies that are associated with myopia development are also found.

Another example is AMD, a leading cause of irreversible blindness in individuals over 65 years of age in developed countries across the world. AMD has considerable heterogeneities in prevalence rates among reported studies of different ethnic populations (Table 16.1). But late AMD is comparable in prevalence between Asian and white populations, in contrast to early AMD, which is less common among Asians, as of dry AMD. Exudative AMD occurs more in Chinese than Caucasians [10]. Polypoidal choroidal vasculopathy (PCV), another serious neovascular retina disease, is three times more common in males than in females in Asians but not in Caucasians [11]. A family-based study of age-related maculopathy has shown that the relative risk of a first degree relative is 6–12 times higher than that of the general population [12].

With gene variants identified for diseases, differential occurrences in their frequencies are discovered. The association of SNP rs1061170 (p. Tyr402His) in the *CFH* gene has been validated in multiple Caucasian populations. It is a common SNP in Caucasians, with a MAF greater than 0.2. However, in East Asian populations, it is an uncommon SNP, with MAF between 0.022 and 0.073 in Japanese and Chinese (HapMap project), respectively. It was initially found not to be associated AMD in these two populations [3, 13]. Later, in a meta-analysis of 13 case-control studies involving 3973 subjects in East Asians, the pooled data revealed a significant association of AMD with rs1061170 [14], but not in any of the individual studies. The statistical power in individual studies was thus inadequate to show the association due to sample sizes and small MAF. Notably, other SNPs in *CFH* are associated with AMD in East Asians, such as the coding variant rs800292 (p.Ile62Val) [3, 13] and the promoter SNP rs3753394 [3]. They are more common in Chinese than Caucasians.

Such ethnic diversity is also found in the *CAV1-CAV2* locus for primary open-angle glaucoma (POAG). SNP rs4236601 at the *CAV1-*

Table 16.1 Prevalence of early and late age-related macular degeneration in different cohort studies

Study	Year	Age	Number	Prevalence (%)	
				Early AMD	Late AMD
Asian population					
Shihpai Eye Study (Taiwan)	1999–2000	≥65	1361	9.2	1.9
Beijing Eye Study (China)	2001	≥40	4439	1.4	0.2
MESA (Chinese American, USA)	2002–2004	45–85	699	3.6	1.0
Handan Eye Study (China)	2006–2007	≥40	5361	3.7	0.1
Hisayama Study (Japan)	1998	≥50	1486	12.7	0.9
Funagata Study (Japan)	2000–2002	≥35	1625	3.5	0.5
Japanese in Brazil	2002–2003	≥60	478	13.4	1.3
Andhra Pradesh Eye Disease Study (India)	1996–2000	≥40	3723	8.9	1.8
India Eye Study (India)	2002–2003	50–79	1443	5.7	1.4
SiMES (Singapore)	2004–2006	40–79	3265	3.5	0.3
The Thailand National Survey of Visual Impairment (Thailand)	2006–2007	≥60	10,788	2.7	0.3
White population					
MESA (Whites, USA)	2002–2004	45–85	2315	4.8	0.6
The European Eye Study		≥65	4753		
The Age, Gene/Environment Susceptibility Reykjavik Study (Iceland)	2002–2006	66–91	5272	12.4	5.3
National Health and Nutrition Examination Survey (non-Hispanic white population, USA)	2005–2008	≥60	1579	11.6	2.6

CAV2 locus was associated with POAG in the Icelandic population in the initial GWAS phase. This association was replicated in other Caucasian cohorts and two Chinese cohorts. The MAF of this SNP was much higher in Caucasians (>0.2) than in Chinese (<0.02), while the odds ratio (OR) was greater in Chinese (OR 3.33–5.42, compared to 1.27 in Caucasians) [15, 148]. However, there is no haplotype-tagging SNP analysis of the *CAV1-CAV2* locus in POAG reported in Chinese. Whether there is a common SNP in this locus, other than rs4236601, is responsible for the association signal detected on the *CAV1-CAV2* locus remains to be investigated. In a subsequent POAG GWAS in Chinese, SNPs at *CAV1-CAV2* were not pinpointed in the GWAS phase [16]. To reveal associated loci that have major effects in Chinese requires GWAS using Chinese subjects in the initial phase.

Differential occurrence profiles of mutations in eye disease genes among Chinese are also known in Mendelian forms [17]. For example, in the *myocilin* (*MYOC*) gene, a disease-causing gene for the Mendelian form of POAG, multiple mutations have been reported in Caucasians (e.g. p.Arg82Cys, p.Glu352Lys and p.Gln368Stop) [18–20]. But they are not detected in Chinese. Rather, different mutations in Chinese are identified, such as p.Gln19His and p.Arg91Stop, p.Glu300Lys, p.Ser313Phe and p.Tyr471Cys [21, 22].

16.3 Spectrum of Age-Related Eye Diseases in Asian Populations

The age-related eye diseases of macular degeneration, glaucoma, diabetic retinopathy and cataracts are the major causes of global visual impairment and blindness. They are responsible for 49.2% of blindness and 25.6% of visual impairment worldwide [23].

16.3.1 Age-Related Macular Degeneration

In Asian populations aged more than 30 years, the prevalence of AMD varies, ranging from

0.4% in Funagata to 12% in Hisayama with a summary estimate of 6.8% (3.14–13.94%) [24]. Macular degeneration is responsible for 19.5%, 13.3%, 6.9%, 2.6% and 5.9% of total blindness in Central, East, South, and Southeast Asia, respectively [23]. In Asians, PCV is a predominant subtype of exudative AMD in contrast to choroidal neovascularization secondary to AMD in Western populations [25]. In the Beijing Eye Study, the overall prevalence of PCV was 0.3% [26]. Most of the epidemiological studies on PCV only reported the prevalence of PCV in neovascular AMD with an estimate ranging from 9 to 61.1% in Chinese, Japanese, and Korean populations [27].

16.3.2 Glaucoma

Primary open-angle glaucoma (POAG) has an overall summary estimate of 2.3% in Asians [28]. Among all glaucoma patients, about 26% are angle-closure glaucoma (ACG), which accounts for about half of the cases blinded by glaucoma [29]. The prevalence of PACG also varies in Asian populations, ranging from 0 to 4.8% with the highest prevalence in Indian and a summary estimate of 1.1% in Asian populations as a whole [28]. Altogether, glaucoma contributes to 11.7%, 12.0%, 5.4%, 4.7% and 5.6% of blindness in Central, East, South, and Southeast Asia, respectively [23].

16.3.3 Diabetic Retinopathy (DR)

DR is becoming a major public health problem in many countries including China and India [30]. Reported prevalence of DR varied from 10 to 61% (median 27.9%) in patients with known diabetes and from 1.5 to 31% (median 10.5%) in newly diagnosed diabetes [30]. DR leads to 4.3%, 4.0%, 1.1%, 2.8% and 1.4% of blindness in Central, East, South and Southeast Asia, respectively [23].

16.3.4 Age-Related Cataract

Cataract is a major cause of global blindness accounting for more than 20 million bilaterally blind subjects and about 94 million visually impaired subjects [31]. In Asia, cataract contributes to 13.1–42.0% of blindness in different regions [23]. The age-standardized cataract prevalence was reported to be 30.4%, 37.8% and 33.1% in Chinese, Malays, and Indians, respectively. It is about 2 times more common and begins about 10 years earlier in Asians than in Caucasian patients [27].

16.4 Mutations in Eye Disease Genes Specific to Chinese

Over the past few years, remarkable progress has been made to identify genetic loci for AMD. Recently, a whole-exome sequencing study was performed in Chinese and detected a missense variant p.Val379Met (rs7739323) in the *UBE3D* gene to be associated with AMD, which indicated a potential link between AMD and the ubiquitin-proteasome system [8]. In another exome-wide study, a Chinese- and East Asian-specific mutation p.Asp442Gly (rs2303790) in the *CETP* gene was identified for AMD [7]. Exome-based studies are thus proved to be useful to detect coding variants for AMD.

For glaucoma, at least 28 genetic loci have been identified for POAG in different populations. Among them, the *MYOC* gene is among the most reported. At least 43 *MYOC* mutations have been identified, but together they account for less than 5% of all POAG patients [17, 32]. Mutation profiles of *MYOC* vary among different populations (Table 16.2). The variant p.Gln368* is the most prevalent *MYOC* mutation in Caucasians. It has been detected in Caucasians, African Americans, and Indians, but not in East Asians such as Japanese and Chinese. A different pattern of *MYOC* sequence variants exists in Chinese when compared with other populations. For example, in a

Table 16.2 Mutations of the *MYOC* gene in primary open-angle glaucoma across different populations

No.	Sequence change	Exon	Caucasian				African		Indian	Mexican	Japanese	Chinese
			USA	Australia	Canada	Germany	African American	South African				
1	p.Arg7His	1								Y[a]		
2	p.Gln19His	1										Y[a]
3	p.Arg45*	1									Y[a]	
4	p.Gln48His	1							Y[a]			
5	17 bp dup 56–72 bp	1					Y[a]					
6	p.Arg76Lys	1				Y[a]						
7	p.Arg82Cys	1	Y[a]	Y[a]								
8	p.Arg91*	1										Y[a]
9	p.Cys245Tyr	3										Y[a]
10	p.Thr256Met	3							Y[a]			
11	p.Trp286Arg	3	Y[a]									
12	p.Thr293Lys	3	Y[a]			Y[a]	Y[a]					
14	p.Gln297His	3									Y[a]	
15	p.Glu300Lys	3										Y[a]
16	p.Ser313Phe	3										Y[a]
17	p.Ser331Thr	3							Y[a]			
18	p.Glu352Lys	3	Y[a]		Y[a]		Y[a]					
19	p.Thr353Ile	3							Y[a]		Y[a]	
20	p.Pro361Ser	3	Y[a]									
21	p.Ala363Thr	3									Y[a]	
22	p.Gly364Val	3	Y[a]									
23	p.Pro370Leu	3							Y[a]			
24	p.Gly374Val	3						Y[a]				
25	p.Gln368*	3	Y[a]	Y[a]	Y[a]		Y[a]		Y[a]			
26	p.Pro370Leu	3							Y[a]			
27	p.Thr377Met	3	Y[a]	Y[a]								
28	p.Ser393Arg	3					Y[a]					
29	p.Lys398Arg	3								Y[a]		
30	p.Gly399Asp	3							Y[a]			
31	p.Ala427Thr	3							Y[a]			
32	p.Tyr437His	3	Y[a]									

33	p.Ala445Val	3		Y[a]		Y[a]						
34	1 bp del codon 453	3					Y[a]					
35	p.Ile465Met	3									Y[a]	
36	p.Arg470Cys	3	Y[a]									
37	p.Tyr471Cys	3										Y[a]
38	p.Ile477Asn	3	Y[a]									
39	p.Asn480Lys	3							Y[a]			
40	p.Pro481Thr	3	Y[a]									
41	p.Pro481Leu	3					Y[a]					
42	p.Glu483*	3			Y[a]							
43	p.Lys500Arg	3						Y[a]				

[a]Y indicates that the variant was only detected in cases or controls in the case-control studies, suggesting that they are likely risk or protective mutations

mutational screening study involving 201 unrelated POAG Chinese patients and 291 controls, 6 novel amino acid changes were found, p.Ala17Ser, p.Leu95Ppr, p.Leu215Pro, p.Glu300Lys, p.Glu414Lys and p.Tyr471Cys [33]. Of these, p.Glu300Lys and p.Tyr471Cys were found only in POAG patients, accounting for about 1.5% of POAG. A nonsense mutation p.Arg46* was not associated with POAG, suggesting that the reduced amount of MYOC predicted to result from this truncation does not affect risk of glaucoma.

16.5 Whole-Genome Association Studies for Age-Related Eye Diseases

GWAS has been providing massive amount of information at the genome level. Thus, it is a major source of genetic discoveries in age-related eye diseases [1]. More than 60 GWAS on eye diseases have been published since 2005 and the number keeps growing (A Catalog of Published Genome-Wide Association Studies; http://www.genome.gov/gwastudies/, accessed November 11, 2015). Most GWAS were about age-related eye diseases, such as AMD [1, 2, 6, 7, 34–47], glaucoma [15, 16, 48–59], diabetic retinopathy (DR) [60–64] and cataract [65]. In the Asian populations, the first GWAS was published in 2006 in Chinese about AMD [2]. By the end of November 2015, there have been five GWAS on AMD [2, 7, 45–47], eight on POAG [16, 53–59], one on PACG [66], three on DR [62–64], and one on age-related cataract [65], all involved Asian study subjects in the initial GWAS discovery phase (Table 16.3).

16.5.1 Age-Related Macular Degeneration

Globally, 15 GWAS have been published on AMD [1, 2, 7, 36–47], and 5 were from Asian populations, including Chinese [2, 7] and Japanese [45–47]. *CFH*, the first AMD-associated gene identified by GWAS [1], linked the complement pathway with AMD pathogenesis. In the promoter region of another gene, *HTRA1*, SNP rs11200638 was strongly associated with exudative AMD in Chinese [2], subsequently confirmed in other GWAS and replication studies in Chinese [3, 67, 68], Japanese [69–71], Korean [72], and Caucasians [73]. Subsequent GWAS have identified *C2*/*CFB* (rs429608) [36], *C3* (rs2230199) [36, 38], *LIPC* (rs10468017) [36, 40], *CETP* (rs3764261) [7, 36] and *TIMP3* (rs9621532) [36]. In a large-scale GWAS, which involved more than 17,100 advanced AMD cases and 60,000 controls mainly of European ancestries, a total of 19 loci for AMD were identified, which together explain 15–65% of total genetic contribution to AMD [6]. Significant associations of *CFH*, *ARMS2*/*HTRA1*, *C3*, *C2*/*CFB* and *CETP* have been repeatedly reported in both GWAS and replication studies in various populations [1, 36–38, 41, 44]. But some variants were discovered only in East Asian populations: *CETP* (rs2303790) [7] and *REST-C4orf14-POLR2B-IGFBP7* (rs1713985, Japanese only) [46] and *C6orf223* (p.Ala231Ala), *SLC44A4* (p.Asp47Val) and *FGD6* (p.Gln257Arg) for Chinese [7]. In a recent GWAS, *MMP20* (rs10895322) was found associated with the lesion size in neovascular AMD in Japanese [45]. Associations in other ethnic populations are yet to be investigated.

As for PCV, more than 60 genetic studies have been published. But there is no GWAS on PCV. It is more prevalent in Asians. Thus, there may be an Asian-specific genetic profile. In a recent systematic review and meta-analysis, 31 SNPs in 10 genes/loci, which had been associated with AMD, were also associated with PCV. Moreover, 12 SNPs at the *ARMS2-HTRA1* locus showed significant differences between PCV and neovascular AMD [74]. The genetic components of neovascular AMD and PCV are, at least partially, different.

16.5.2 Glaucoma

As of November 2015, 15 GWAS have been published on POAG and its subtypes (normal-tension and high-tension POAG) in various

Table 16.3 Genome- and exome-wide association studies of age-related eye diseases involving Asian subjects as initialization cohorts

No.	Ethnicity	Disease phenotype	Cases	Controls	SNP	Adjacent gene(s)	Author (year)
Age-related macular degeneration							
1	Chinese	AMD	96	130	rs11200638	*ARMS2/HTRA1*	Dewan A et al. (2006)
2	Japanese	Wet AMD	100	190	rs10490924	*ARMS2/HTRA1*	Goto A et al. (2009)
		PCV	100	190	rs10490924	*ARMS2/HTRA1*	
3	Japanese	Wet AMD	1536	18,894	rs800292	*CFH*	Arakawa S et al. (2011)
					rs3750847	*ARMS2*	
					rs13278062	*TNFRSF10A-LOC389641*	
					rs1713985	*REST-C4orf14-POLR2B-IGFBP7*	
4	Japanese	Neovascular lesion size	1146		rs10895322	*MMP20*	Akagi-Kurashige Y et al. (2015)
					rs2284665	*ARMS2/HTRA1*	
5	East Asians	AMD	6345	15,980	rs2303790	*CETP*	Cheng CY et al. (2015)
					p. Ala231Ala	*C6orf223*	
					p.Asp47Val	*SLC44A4*	
					p. Gln257Arg	*FGD6*	
6	East Asians	AMD	3988	8495	p. Val379Met	*UBE3D*	Huang LZ et al. (2015)
Primary open-angle glaucoma							
1	Japanese	POAG	418	300	rs2499601	*ZP4*	Nakano M et al. (2009)
					rs540782	*ZP4*	
					rs547984	*ZP4*	
					rs693421	*ZP4*	
					rs7081455	*PLXDC2*	
					rs7961953	*DKFZp762A217*	
2	Japanese	NTG	305	355	rs3213787	*SRBD1*	Meguro A et al. (2010)
					rs735860	*ELOVL5*	
3	Japanese	POAG (NTG)	833	686	rs518394	*CDKN2B-AS1*	Nakano M et al. (2012)
					rs523096	*CDKN2B-AS1*	
					rs564398	*CDKN2B-AS1*	
					rs7865618	*CDKN2B-AS1*	

(continued)

Table 16.3 (continued)

No.	Ethnicity	Disease phenotype	Cases	Controls	SNP	Adjacent gene(s)	Author (year)
4	Japanese	POAG	1394	6599	rs10483727	*SIX1-SIX6*	Osman W et al. (2012)
					rs1063192	*CDKN2B*	
					rs7588567	*NCKAP5*	
5	Japanese	NTG	437	745	rs523096	*CDKN2B-AS1*	Takamoto M et al. (2012)
6	Japanese	NTG	717	391	rs678350	*HK2*	Shi D et al. (2013)
					rs2033008	*NCK2*	
7	Chinese	HTG	1007	1009	rs2487032	*ABCA1*	Chen Y et al. (2014)
					rs3785176	*PMM2*	
8	Asians, Caucasians, Africans	POAG	12,677	36,526	rs2157719	*CDKN2B-AS1*	Li Z et al. (2015)
					rs1192415	*TGFBR3*	
Primary angle-closure glaucoma							
1	Chinese	PACG	3771	18,551	rs11024102	*PLEKHA7*	Vithana EN et al. (2012)
					rs3753841	*COL11A1*	
					rs1015213	*PCMTD1-ST18*	
Diabetic retinopathy							
1	Chinese	Type 2 diabetes	174	575	rs12092121	*MYSM1*	Huang YC et al. (2011)
					rs2811893	*MYSM1*	
					rs4470583	n.a.	
					rs13163610	n.a.	
					rs17376456	n.a.	
					rs1571942	*PLXDC2*	
					rs12219125	n.a.	
					rs4838605	*ARHGAP22*	
					rs11101355	*ARHGAP22*	
					rs11101357	*ARHGAP22*	
					rs4462262	n.a.	
					rs2038823	*HS6ST3*	
2	Cohort-1: ChineseCohort-2: Hispanics	Type 2 diabetes	437	570	rs4668142	*LRP2-BBS5*	Sheu WH et al. (2013)
					rs2380261	*ARL4C-SH3BP4*	
					rs9543976	*TBC1D4-COMMD6-UCHL3*	
3	Japanese	Type 2 diabetes	837	1149	rs9362054	*RP1-90 L14.1*	Awata T et al. (2014)
Age-related cataract							
1	Mixed Asians	Nuclear cataract	7140		rs7615568	*KCNAB1*	Liao J et al. (2014)
					rs11911275	*CRYAA*	

n.a. indicates that there are no adjacent genes reported for the SNP

populations [15, 16, 43, 48–59]. More than 50 SNPs in 20 loci have been associated with POAG: *ABCA1* [16, 49], *CAV1*/*CAV2* [15], *CDKN2B-AS1* [48, 52, 57–59, 75], *SIX1*/*SIX2* [59], 16q12 [76], *AFAP1* [49], *C6orf105* [50], *C7* [43], *CNTNAP4* [76, 77], *DKFZp762A217* [56], *ELOVL5* [55], *GAS7* [78], *GMDS* [49], *LY86-AS1* [76], *NCKAP5* [59], *NEDD9* [50], *NTM* [76], *PLXDC2* [56], *PMM2* [16], *SRBD1* [55], *TMCO1* [48], *TNN* [76] and *ZP4* [56]. The majority of POAG subjects in the published GWAS were of high-tension POAG. Among them, 13 genes and 19 SNPs were identified from Chinese [16] and Japanese [54–59]. Overlapping SNPs between GWAS from Asian and non-Asian populations mainly clustered in two loci, i.e., *CDKN2B-AS1* and *SIX1*/*SIX6*. As for PACG, a GWAS was reported in 2012 in the Chinese population, identifying three genetic loci (*PLEKHA7*, *COL11A1*, and the *PCMTD1-ST18* locus) [66]. SNPs in these three loci have been replicated in study cohorts from Chinese [16, 79], Caucasians [80] and Indian [81, 149].

16.5.3 Diabetic Retinopathy

So far five GWAS on DR have been published as full-text articles [60–64] with sample sizes ranging from 286 [60] to 2829 [61]. Among them, three were from Chinese [63, 64] and Japanese populations [62]. A total of 35 SNPs in or near 26 genes and 14 intergenic SNPs have been identified. None of the reported SNPs in Asian populations have been replicated in independent cohorts. In a non-Asian study, the top SNPs from the GWAS of Grassi et al. [61] were retested in a separate cohort, but it was found to have no genome level of significance for proliferative DR when combining the data from the GWAS and the replication study [82]. In addition, the findings have not been replicated in follow-up stuides using separate cohorts.

16.5.4 Age-Related Cataract

As of November 2015, the only GWAS on age-related cataract published in the Asian population involved Chinese, Malays and Indian [65]. Two genetic loci, *KCNAB1* (rs7615568) and *CRYAA* (rs11911275), have been discovered in Malay and Indian populations and replicated in Singapore and Beijing Chinese populations. However, these signals remain to be replicated in other Asian and non-Asian populations, such as Japanese, Caucasians or Africans.

16.5.5 Others

GWAS have also been reported in Chinese populations for myopia and refractive errors [83–97], Behcet's disease [98–103] and Vogt-Koyanagi-Harada syndrome (VKH) [104].

16.6 Whole-Genome Association Studies for Endophenotypes

Ocular endophenotypes are the anatomical characteristics of the eyes. Some are recognized as risk factors for age-related eye diseases, such as central cornea thickness (CCT), anterior chamber depth (ACD), intraocular pressure (IOP), and optic disc parameters. As of November 2015, a total of 21 GWAS on CCT [50, 76, 85, 105–109], ACD [110], IOP [50, 54, 78, 111–115] and optic disc parameters [50, 75, 116–119] have been published. Among them, seven GWAS originated or involved Asian populations, such as Chinese, Malays and Indian (Table 16.4) [54, 106, 109, 110, 117–119]. Besides, multi-ancestry GWAS involving Asian populations have been performed, prioritizing additional genetic loci for further investigations [54, 106, 109, 110, 117, 118].

16.6.1 Central Corneal Thickness

CCT is a highly heritable trait [120, 121]. A thinner CCT is a proved risk factor for progression from ocular hypertension to POAG [122]. Some genes associated with CCT are also associated with increased risk of keratoconus, such as *FOXO1* and *FNDC3B* [109]. In the eight

Table 16.4 GWAS of endophenotypes of age-related eye diseases involving Asians as initialization cohorts

No.	Ethnicity	Ocular trait	Sample size	SNP	Adjacent gene(s)	First author (year)
Central corneal thickness (CCT)						
1	Mixed (Indian, Malays)	CCT	5080	rs96067	*COL8A2*	Vithana EN et al. (2011)
		CCT	5080	rs1536478	*RXRA-COL5A1*	
2	Mixed Asians and Caucasians	CCT	20,020	rs96067	*COL8A2*	Lu Y et al. (2013)
		CCT	20,020	rs7606754	*COL4A3*	
		CCT	20,020	rs4894535	*FNDC3B*	
		CCT	20,020	rs7620503	*TBL1XR1-KCNMB2*	
		CCT	20,020	rs3931397	*NR3C2*	
		CCT	20,020	rs2307121	*ADAMTS6*	
		CCT	20,020	rs1538138	*FAM46A-IBTK*	
		CCT	20,020	rs11763147	*VKORC1L1*	
		CCT	20,020	rs4718428	*C7orf42*	
		CCT	20,020	rs1324183	*MPDZ-NF1B*	
		CCT	20,020	rs1007000	*LPAR1*	
		CCT	20,020	rs1536482	*RXRA-COL5A1*	
		CCT	20,020	rs7044529	*COL5A1*	
		CCT	20,020	rs11145951	*LCN12-PTGDS*	
		CCT	20,020	rs7090871	*ARID5B*	
		CCT	20,020	rs4938174	*ARHGAP20-POU2AF1*	
		CCT	20,020	rs1564892	*GLT8D2* (5′)	
		CCT	20,020	rs1034200	*FGF9-SGCG*e	
		CCT	20,020	rs2721051	*FOXO1* (3′)	
		CCT	20,020	rs785422	*TJP1* (5′)	
		CCT	20,020	rs12913547	*SMAD3*	
		CCT	20,020	rs6496932	Near *AKAP13* (5′)	
		CCT	20,020	rs930847	*LRRK1*	
		CCT	20,020	rs752092	*CHSY1*	
		CCT	20,020	rs9938149	*BANP-ZNF469*	
		CCT	20,020	rs12940030	*HS3ST3B1-PMP22*	

(continued)

Table 16.4 (continued)

No.	Ethnicity	Ocular trait	Sample size	SNP	Adjacent gene(s)	First author (year)
Anterior chamber depth (ACD)						
1	Mixed Asians	ACD	5308	rs1401999	*ABCC5*	Nongpiur ME et al. (2014)
Intraocular pressure (IOP)						
1	Mixed Asians and Caucasians	IOP	35,296	rs6445055	*FNDC3B*	Hysi PG et al. (2014)
		IOP	35,296	rs2472493	*ABCA1*	
		IOP	35,296	rs8176693	*ABO*	
		IOP	35,296	rs747782	*11p11.2*	
		IOP	35,296		*TMCO1*, *CAV1*, *GAS7*	
Optic disc parameters						
1	Mixed Asians and Caucasians	Optic disc area	18,216	rs9607469	*CARD10*	Khor CC et al. (2011)
	Mixed Asians and Caucasians	Optic disc area	18,216	rs1192415	1p22 (117 kb to *CDC7*)	
	Mixed Asians and Caucasians	Optic disc area	18,216	rs7916697	*ATOH7*	
2	Mixed Asians and Caucasians	Vertical cup/disc ratio	27,878		*COL8A1*, *DUSP1*, *EXOC2*, *PLCE1*, *ADAMTS8*, *RPAP3*, *SALL1*, *BMP2*, *HSF2*, and *CARD10*	Springelkamp H et al. (2014)
3	Mixed Asians and Caucasians	Optic disc area	24,089		10 new loci: *CDC42BPA*, *F5*, *DIRC3*, *RARB*, *ABI3BP*, *DCAF4L2*, *ELP4*, *TMTC2*, *NR2F2*, and *HORMAD2*	Springelkamp H et al. (2015)
		Optic cup area	24,089		10 new loci: *DHRS3*, *TRIB2*, *EFEMP1*, *FLNB*, *FAM101*, *DDHD1*, *ASB7*, *KPNB1*, *BCAS3*, and *TRIOBP*	
Axial length (AL) and corneal curvature (CC)						
1	Mixed Caucasians and Asians	AL	20,747		*ZC3H11B*, *RSPO1*, *C3orf26*, *LAMA2*, *GJD2*, *ZNRF3*, *CD55*, *MIP*, and *ALPPL2*	Cheng CY et al. (2013)
2	Mixed Chinese and Malays	AL	4944	rs4373767	*ZC3H11B*	Fan Q et al. (2012)
3	Japanese	AL and CC	15,168	rs10453441	*WNT7B*	Miyake M et al. (2015)

GWAS that identified 31 loci for CCT, 12 were from Asian populations [106, 109], and six genes/loci have been repeatedly reported by GWAS in multiple ethnic groups, including *AKAP13* [105, 109], *COL5A1* [105, 109], *COL8A2* [106, 109], *RXRA-COL5A1* [106, 108, 109], *FOXO1* [85, 109] and *ZNF469* [85, 105, 107]. The largest GWAS tested more than 20,000 subjects of European or Asian origins [109], and ten new loci were identified.

16.6.2 Anterior Chamber Depth

The only GWAS on ACD tested more than 5300 subjects of Asian descent. The gene for ACD, *ABCC5* (rs1401999), was also associated with risk of PACG [110], providing evidence for shared pathways between abnormal anterior chamber development and the pathogenesis of PACG.

16.6.3 Intraocular Pressure

Although IOP elevation is a result of a reduced aqueous humor outflow facility due to multiple factors, genetic factors also seem to play a role [123, 124]. The first GWAS on IOP was from Caucasians, in which *GAS7* (rs7555523) and *TMCO1* (rs7555523) were identified [78]. Other GWAS reported 11 loci in Caucasian [111–115] and Asian [54] populations, such as *GLCCI1*/*ICA1*, *CAV1*/*CAV2* and *FAM125B*. In a multi-ancestry GWAS involving Caucasians, Chinese, Malays and Indian, most of the previously reported loci have been replicated, with additional genes *FNDC3B*, *ABCA1*, *ABO* and the 11p11.2 locus [54]. No GWAS of IOP is solely based on the Asian populations.

16.6.4 Optic Disc Parameters

Among the six GWAS on genetic factors for the optic disc area [75, 116, 117, 119], cup area [119] and VCDR [75, 118], three involved both Asian and European populations, highlighting 11 genes/loci for optic disc area (i.e., *CARD10*, *CDC42BPA*, *F5*, *DIRC3*, *RARB*, *ABI3BP*, *DCAF4L2*, *ELP4*, *TMTC2*, *NR2F2* and *HORMAD2*), ten for cup area (i.e., *DHRS3*, *TRIB2*, *EFEMP1*, *FLNB*, *FAM101*, *DDHD1*, *ASB7*, *KPNB1*, *BCAS3* and *TRIOBP*), and ten for VCDR (*COL8A1*, *DUSP1*, *EXOC2*, *PLCE1*, *ADAMTS8*, *RPAP3*, *SALL1*, *BMP2*, *HSF2* and *CARD10*) [117–119]. Most of the loci originally identified in Caucasian populations have been replicated in Asian populations, including *ATOH7*, *CDC7* and *SIX6*.

16.6.5 Axial Length and Corneal Curvature

There are three GWAS on AL [83, 84, 125] and CC [125, 126], two from Chinese [83, 84] and one from Japanese [125]. Nine loci have been identified in Asians for AL, including *ZC3H11B*, *RSPO1*, *C3orf26*, *LAMA2*, *GJD2*, *ZNRF3*, *CD55*, *MIP* and *ALPPL2* [83, 84], and one locus, *WNT7B*, for both AL and CC [125].

16.7 Ethnic-Specific Genome Information

Genome-wide investigation of single nucleotide variants (SNVs) and copy number variants (CNVs) has provided insights into global human genetic diversity, including demography and migration patterns of human populations, ancient DNA, *de novo* mutation rates, and the relative deleteriousness and frequency of coding mutations [127, 128]. Even among Chinese, the major ethnic group, Han, is intricately substructured, with the main observed clusters corresponding roughly to northern Han, central Han and southern Han [129, 130]. Genetic differentiation among these clusters is sufficient to lead to an inflated rate of false-positive results if sampling was done across different clusters [129]. In such case, significant differences among Han Chinese subpopulations should be carefully explained in case they are also detected in association studies. In GWAS, different

methods, such as the genomic control method [2] and principal component analysis (PCA) [16] have been widely adopted to address the potential population stratification and proved to be useful in reducing the risk of false positives.

In two major eye diseases, AMD and glaucoma, some Chinese-specific genetic features have been obtained through GWAS.

16.7.1 Age-Related Macular Degeneration

Among the 19 AMD loci identified in the large-scale GWAS that account for 15–65% of total genetic contribution to AMD in Caucasians [6], some have been replicated in Chinese (*ARMS2-HTRA1*, *CFH*, *C2-CFB*, *C3*, *APOE*, *CETP* and *LIPC*), with different associations. *APOE* has been excluded as a major associated gene for AMD in Chinese [131–133]. Althought *CFH* rs1061170 was significantly associated with AMD both in Chinese [134] and Caucasians, *CFH* rs800292, but not rs1061170, was proved to be the major AMD-associated SNP in Chinese [3, 13]. In addition, another meta-analysis showed that *SERPING1* is not a major genetic component of AMD in East Asians but for AMD in Caucasians [135]. Apart from common gene variants, a rare variant in *CFH*, p. Arg1210Cys, is strongly associated with AMD in Caucasians [136] but not in Chinese [4, 137]. Also, a *CFHR1-CFHR3* deletion was associated with a reduced risk of AMD in Caucasians [138, 139], but it was non-polymorphic in Chinese [140]. Rare variants in *CFI*, *C3* and *C9*, have been identified in AMD in Caucasians [136] but not in Chinese [7]. Such variants may be too rare and require a much larger sample size to be detected in Chinese, or they may be genuinely specific to Caucasians. In contrast, some variants were discovered only in Chinese populations, including *CETP* (Asp442Gly, rs2303790) [7], *C6orf223* (p. Ala231Ala), *SLC44A4* (p.Asp47Val), *FGD6* (p. Gln257Arg) [7] and p.Val379Met (rs7739323) in *UBE3D*.

16.7.2 Glaucoma

POAG is a complex disease with both Mendelian and multifactorial forms. To date, linkage analysis and sequencing study have reported nine genes and 28 genetic loci for POAG [141, 142]. Besides, more than 120 genes have been reported in genetic association studies, including GWAS, such as *ABCA1* [49, 143], *CAV1*/*CAV2* [15], *CDKN2B-AS1* [48, 52, 54, 57–59], *SIX1*/*SIX6* [59] and *TMCO1* [48, 78]. Association of the *CDKN2B-AS1* locus with POAG has been confirmed by six GWAS [48, 52, 57–59, 75]. Replication studies in Asian populations have confirmed some of these GWAS loci, such as *CAV1*/*CAV2*, *CDKN2B-AS1* and *SIX1*/*SIX6*. There are ethnic differences in the allele frequencies and association profiles. Notably, the minor allele frequencies and effect sizes of rs4236601 in the *CAV1*/*CAV2* locus were different across populations. It is a common SNP in Caucasians, with the minor allele A presented in 20–28% of the control subjects, conferring an odds ratio of 1.1–1.3 [144–146]. In contrast, rs4236601 is rare in Chinese and Korean, with the allele A presented in less than 1% of the population, but conferring a higher OR of 2.9–5.5 [15, 147–148].

16.8 Future Prospects

Studies on Chinese cohorts have helped revealing gene variants for diseases, especially those of differentially high occurrences in Chinese. GWAS does require large sample size. Detailed and accurate documentation of clinical information have been proved to be pivotal. The logistic and financial demands are huge. As the development of new sequencing techniques and lowering of the cost per sample, exome array-based association studies, whole-exome sequencing, and even whole-genome sequencing studies are emerging. This will supplement a group of new, high-impact (but usually rare), population-specific, and functional variants to the genetic components of eye diseases. Also, genome-wide gene-gene and gene-environmental studies will be providing gene variants with epistatic effects. Compared with

traditional GWAS, the scale of these studies would be even larger, which in turn require sufficient funding, large number of study samples, detailed documentation of clinical information, replications and validations of significant rare variants, and expert bioinformaticians for effective data management and processing. International collaboration will play a very important role in such studies.

With the emerging of new loci for diseases and endophenotypes from the genome-wide platforms, candidate gene analysis will keep playing an important role in the genetics of eye diseases. First, replication studies on these new candidate genes in specific study cohorts will generate useful information for a better understanding of the roles of the genes. Second, as the associated SNPs identified in GWAS are usually not the causal variants for the associations, regional fine-mapping (e.g., haplotype-tagging SNP analysis) and/or targeted resequencing will provide new information for pinpointing the key variants. Furthermore, in candidate gene analyses, the genes identified in GWAS can be correlated with various clinical subtypes and endophenotypes of eye diseases. Small- to medium-sized research groups will play a good role in candidate gene analyses.

In the near future, more gene variants are expected to be identified, and genome-wide studies will eventually lead the way to personalized medicine. Discoveries of gene information have enabled analysis of gene-gene interactions and gene-environment effects. Another obvious consequence of mapping disease genes is to study the biological relevance that would lead to understanding of disease pathogenesis and devise effective treatments.

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Clinical Genetics of Retinoblastoma: An Asian Perspective

17

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Abstract

Retinoblastoma is a cancer of the eye that affects children under the age of 5 years. Mutations in the *RB1* gene cause retinoblastoma. Identification of the *RB1* germline status of a patient allows differentiation between sporadic and heritable retinoblastoma variants. Application of this knowledge is crucial for assessing short-term (risk of additional tumors in the same eye and other eye) and long-term (risk of non-ocular malignant tumors) prognosis and offering cost-effective surveillance strategies. Accurate risk assessment and successful counseling allow families to consider reproductive options. Access to certified laboratories providing high-quality and reliable genetic testing for *RB1* is limited in Asian countries.

Keywords

Retinblastoma • Retinoma/retinocytoma • Genetic testing • Genetic counseling • *RB1* gene mutation

17.1 Introduction

Retinoblastoma is a cancer of the eye that affects children under the age of 5 years [1, 2]. Mutations in the *RB1* gene cause retinoblastoma [3–5]. Mutations in both *RB1* alleles within the precursor retinal cell are essential, with one mutation that may be germline or somatic and the second one that is always somatic. Mutations in the *RB1* gene cause retinoblastoma, with one mutation that may be either germline or somatic, and the second mutation that is always somatic. Mutations in both *RB1* alleles within the precursor retinal cell are necessary to cause disease. Identification of the *RB1* germline status of a patient allows differentiation between sporadic and heritable retinoblastoma variants. Application of this knowledge is crucial for assessing short-term (risk of additional tumors in the same eye and other eye) and long-term (risk of

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non-ocular malignant tumors) prognosis and offering cost-effective surveillance strategies [6].

Genetic testing and genetic counseling are, therefore, essential components of care for all children diagnosed with retinoblastoma. Genetic counseling of retinoblastoma families must rely on accurate interpretation of clinical and molecular data in order to achieve the goal of decreasing the burden of this disorder in both the immediate and extended family. In this chapter we discuss the clinically relevant aspects of genetic testing and genetic counseling for a child with retinoblastoma.

17.2 Inheritance Pattern

Retinoblastoma is inherited in a dominant manner. Not every individual who inherits the germline mutation is affected, though. Half of all affected children are estimated to be heritable and carry a germline *RB1* mutation [1]. Each child born to a person with a germline *RB1* mutation has a 50% risk of inheriting the mutation as is characteristic of dominant inheritance. Although 90% of persons with a germline RB1 mutation will develop retinoblastoma (60% multifocal, 30% unifocal) and the remaining 10% will not develop tumors and remain as unaffected carriers, only 10% of children with unilateral or bilateral retinoblastoma have a positive family history.

Knudson first proposed the "two-hit" hypothesis whereby two complementary mutational events are required for the development of retinoblastoma [7]. In heritable retinoblastoma, the first "hit," or mutation, is a germline mutation and present in all cells of the affected child. The second mutation usually occurs in multiple retinal progenitor cells resulting in multifocal or bilateral retinoblastoma. In sporadic (nonheritable) retinoblastoma, there is no germline mutation, and both mutations occur somatically in a single retinal progenitor cell causing unifocal (unilateral) retinoblastoma.

17.3 Clinical Features

Age of diagnosis is earlier in heritable retinoblastoma, at an average age of 15 months, whereas nonheritable cases usually present later with an average age of 24 months [2]. It is assumed that all children with bilateral involvement of retinoblastoma have a germline mutation, even if genetic testing is unable to identify the mutation (which occurs in approximately 5% of cases). In addition, a germline *RB1* mutation is present in 15% of children with unilateral retinoblastoma.

The most common signs of presentation are a white pupil (leukocoria) and strabismus [2]. Clinical signs of advanced intraocular disease include secondary neovascular glaucoma or massive tumor necrosis and intraocular hemorrhage [8]. More advanced tumors could undergo extensive intraocular necrosis leading to an aseptic orbital cellulitis. The tumor appears as a white-yellow lesion arising from the retina. Calcification is a pathognomonic feature of larger tumors and is of diagnostic value when detected by ultrasonography or radiological imaging. In each affected eye, tumors can occur as a single lesion or in multiples (multifocal), with the latter being suggestive of the presence of heritable retinoblastoma [9]. Each lesion can produce "seeds" that disseminate into the vitreous or subretinal spaces. An exudative retinal detachment allows the tumor to seed into the subretinal space. The morphology of seeds in the vitreous can be described as dust, clouds, or pearl-like spheres [10, 11]. These could extend into the anterior chamber.

An invasive tumor may break Bruch's membrane and spread into the predominantly vascular choroid, potentially allowing tumor cells to metastasize via a hematogenous route [12]. Alternatively, the tumor might spread via the lamina cribrosa into the optic nerve, allowing the tumor to access cerebrospinal fluid and the central nervous system. Tumor extending and spreading through sclera can metastasize through lymphatics.

With timely detection, more than 95% of those affected can survive if a multidisciplinary

team provides coordinated and collaborative treatment between specialized centers with appropriate expertise, up-to-date protocols, and modern equipment [13]. The treatments may involve surgery (enucleation), chemotherapy (intravenous, intra-arterial, periocular, or intraocular), focal therapies (laser and cryotherapy), and radiation (brachytherapy, external beam radiation). Treatment is first prioritized ensuring patient survival and then at preserving the eyeball and optimizing visual function.

17.4 Distinct Features of Heritable Retinoblastoma

Children with germline mutations usually present with multifocal or bilateral retinoblastoma. All children with a positive family history of retinoblastoma are considered to have heritable retinoblastoma.

17.4.1 13q Deletion Syndrome

It is estimated that 5–6% of all children with retinoblastoma have an interstitial chromosome deletion or translocation of region 13q14 [14]. Such children may demonstrate dysmorphic features, developmental delay, and psychomotor retardation. The degree of severity correlates with the size of the chromosomal deletion and involvement of contiguous genes. Some of the characteristic facial features associated with this syndrome include thickened and anteverted ear lobes, high and broad forehead, prominent philtrum, short nose, and thick everted lower lip [15]. Testing for chromosome deletions and translocations is typically performed using karyotype and/or chromosomal microarray. With the expanding use of karyotyping and chromosomal microarray in genetic testing for children with mental retardation, 13q deletion syndrome may be identified prior to a diagnosis of retinoblastoma. These children should be followed with frequent eye exams as recommended for all children with heritable retinoblastoma.

17.4.2 Trilateral Retinoblastoma

Occurrence of a primitive neuroectodermal tumor (or a pinealoma) known to occur in the setting of heritable retinoblastoma has been termed as "trilateral" retinoblastoma [16, 17]. These tumors may be located in the pineal gland or suprasellar/parasellar region. The chance of developing trilateral retinoblastoma is less than 0.5% for children with unilateral retinoblastoma, 5 to 13% for bilateral retinoblastoma, and 5 to 15% for familial bilateral retinoblastoma [18]. Unfortunately the median survival for children with trilateral retinoblastoma is only 9 months. Screening recommendations for intracranial malignancies include gadolinium-enhanced magnetic resonance imaging every 6 months until the age of 4.5 years for children with heritable form of retinoblastoma [19].

17.4.3 Second Malignant Neoplasms

The risk of other (non-ocular) malignancies in individuals with heritable retinoblastoma significantly increases over their life span. The most common second cancers include osteosarcoma, soft tissue sarcoma, melanoma, and epithelial cancers. Some studies suggest those with familial retinoblastoma have a greater risk for second cancers compared to those with a de novo *RB1* mutation [20]. Individuals with heritable disease who received external beam radiation therapy have a further increased risk of developing malignancies, especially in the radiation field. Avoidance of DNA-damaging agents such as tobacco, UV radiation, and X-ray is recommended for individuals with heritable retinoblastoma. While there are no guidelines stating periodic imaging for second cancers should be performed, a low threshold for investigation

should exist if any symptoms or signs of a possible cancer arise.

17.4.4 Retinoma/Retinocytoma

A retinoma is a rare, benign variant of retinoblastoma also caused by mutations in the *RB1* gene [21]. Studies have shown that retinoma is a precursor of retinoblastoma. On occasion (4% of the time), retinoma may undergo malignant transformation, and therefore adults and children with these lesions should be followed closely [22]. In contrast to retinoblastoma, retinomas diagnosed in childhood or adulthood are often asymptomatic.

17.4.5 Low-Penetrance Retinoblastoma

Most *RB1* germline mutations are highly penetrant. The penetrance, or chance to develop retinoblastoma with a typical "null" germline mutation, is 90% or higher. However, in a few families, the penetrance is much lower than 90% with high proportion of unilateral retinoblastoma (reduced expressivity) or carriers (no tumors at all, incomplete penetrance) [23]. Mutations associated with low penetrance are typically missense mutations or mutations in the promoter region that produce a low level of Rb protein rather than total absence of protein [24].

17.5 The *RB1* Gene and Function

The *RB1 gene* is a tumor suppressor gene that regulates cell cycle. Located on the long arm of chromosome 13, the gene consists of 27 exons dispersed over 200 kb of genomic DNA [25]. The RB protein regulates the cell cycle by acting as a transcriptional repressor by targeting the E2F transcription factors. Dividing cells are therefore prevented from entering the S phase of mitosis and proceed to unregulated cycles of mitosis.

17.6 RB1 Gene Mutations

Mutations in the *RB1* gene result in a loss of function that inactivates the RB1 protein. The retinoblastoma protein is frequently inactivated by deletions and nonsense mutations [24]. To date, many *RB1* gene mutations have been identified that cause heritable retinoblastoma [3, 5, 24, 26]. Although suspected hotspots have been identified, they account for only 40% of the mutations. The rest of the mutations are scattered throughout the entire gene, most frequently in exons 9, 10, 14, 17, 18, 20, and 23. Children with splicing mutations are often diagnosed at a later age than children with frameshift, nonsense, or missense mutations. The reported mutations in low-penetrance families were commonly those that result in low expression of a normal protein (such as regulatory sequences at the promoter) or cause a partial inactivation of its functions (such as missense and in-frame deletions affecting non-essential sequence motifs).

17.7 $RB1^{+/+}$ $MYCN^{A}$ Tumors

A rare and genetically unique form of retinoblastoma was discovered in which both alleles of the *RB1* gene were normal, and instead expression of the *MYCN* gene was considerably amplified [27]. This form of tumor is clinically indistinguishable from *RB1* mutated tumors. It has exclusively been discovered in 1–3% of children with unilateral retinoblastoma, especially in those under the age of 12 months. Histopathology of these tumors seems to demonstrate undifferentiated cells with large, prominent, multiple nucleoli and necrosis, apoptosis, and little calcification. The histopathology feature of this form of retinoblastoma resembles that of other MYCN-amplified tumors such as neuroblastoma. The characteristics of this type of tumor strongly suggest nonhereditary disease, without risk for retinoblastoma in the other eye, for other malignant cancers throughout life, and risk of familial transmission.

17.8 Genetic Testing

A meticulous and comprehensive approach can detect about 95% of the *RB1* mutations [9]. Identification of the *RB1* mutation reduces overall healthcare expenditures by identifying those children who are at risk for additional intraocular tumors (ipsilateral or contralateral eye), trilateral retinoblastoma, or second malignant tumors, thereby sparing relatives who test negative for the *RB1* germline mutation from unnecessary screening evaluations [5]. It is recommended that genetic testing be conducted in a certified laboratory with demonstrated sensitivity (% of all tested samples in which the mutation(s) are found, resulting in a useful report) and turnaround time to identify *RB1* mutations [13]. The cost of testing is becoming more affordable with advances in genetic testing technology such as next-generation sequencing.

The One RB World website (http://1rbw.org) aims to list all genetic testing laboratories that perform retinoblastoma testing, including their contact information, test sensitivity, and relevant information about their processes. As of July 2015, there were two centers in India, one in China, and none in the rest of Asia. Laboratories may contribute their data directly to the website to be reported.

17.9 Indications for Testing

A clinical diagnosis of retinoblastoma in a child whose germline *RB1* status is not already known is an indication for testing. A child with bilateral disease, with trilateral retinoblastoma, and those with a family history almost certainly carry a germline *RB1* gene mutation, making them at risk for developing second malignant neoplasms. Genetic testing may detect at-risk members of the family who may harbor the *RB1* mutation, including possibility of prenatal testing to the parents. Children with unilateral disease without any family history require genetic testing to determine their risk for additional intraocular tumors (ipsilateral or contralateral eye), trilateral retinoblastoma, or second malignant tumors. Illustrative case studies are presented in this article. The genetic test for the proband was done by Impact Genetics (Toronto, Canada) and for the other family members by the Center for Human Genetics (Bengaluru, India):

Case 1 (Fig. 17.1) A 1.5-year-old girl presented with bilateral retinoblastoma, with her father being affected with unilateral retinoblastoma. The disease was advanced at presentation, as the parents were unaware that their children could inherit the disease. The child died from metastatic disease. During the management of this girl, clinical examination of her younger brother (3 months old) led to the detection the of early-stage retinoblastoma. The *RB1* mutation is now known, and the parents are aware that any future children can be prenatally evaluated to assess for *RB1* mutation.

Case 2 (Fig. 17.2) A 3.5-year-old boy presented with bilateral retinoblastoma. A family history revealed that his mother had been enucleated in early childhood for "trauma" to the eye. Genetic testing confirmed that the proband and his mother carried identical *RB1* gene mutation.

Case 3 (Fig. 17.3) A 2.5-year-old boy with bilateral retinoblastoma presented without family history of the disease. Retinal examination of his father revealed an asymptomatic retinoma. Genetic testing for *RB1* gene mutation identified germline mutation in the proband and his father. The sibling was unaffected by the mutation and therefore not subjected to serial examinations under anesthesia.

17.10 Requirements for Testing

High-quality deoxyribonucleic acid (DNA) is required from the affected proband to detect mutations in the *RB1* gene. Testing for germline *RB1* mutation usually requires a peripheral blood sample or saliva collected using a specifically designed kit. In case these studies do not reveal *RB1* mutation, it is important to preserve tumor

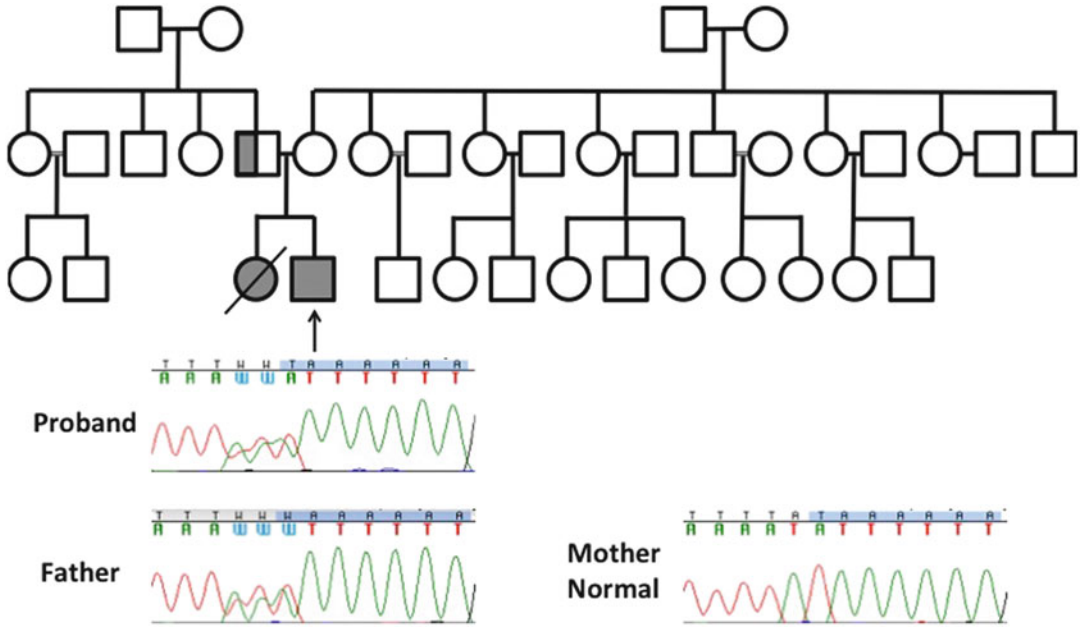

Fig. 17.1 A 1.5-year-old girl presented with bilateral retinoblastoma, with her father being affected with unilateral retinoblastoma. *RB1* gene mutation was detected in the father and younger affected sibling (c.1959A dupA)

tissue so that high-quality DNA can be obtained for subsequent genetic studies. Tumor tissue can be obtained fresh in the operating room (after an enucleation) or in the pathology lab before formalin fixation. Although fresh tumor extraction and flash freezing yield sufficient good-quality tissue for analysis, it needs to be done very carefully using minimally disruptive techniques that will least affect histopathological analysis of an enucleated eye. Two *RB1* mutations should be detected in tumor DNA. If one of these mutations is detected in the blood or saliva sample, the child has germline form of retinoblastoma. If neither mutation is identified in germline DNA, the chance for heritable retinoblastoma is low, but cannot be ruled out due to the chance of low-level mosaicism. Good laboratories should be able to detect mosaicism in as low as 20% of sampled leukocytes. Blood and tissue extraction methods, storage methods, and transportation to the testing laboratory are unique to each laboratory, and it is necessary to discuss them in detail prior to sample collection.

17.11 Laboratory Methods

- DNA sequencing of the exons and intron-exon boundaries identifies approximately 70–75% of all *RB1* mutations [26].
- Multiplex ligation-dependent probe amplification (MLPA) or quantitative multiplex polymerase chain reaction (QM-PCR) is used to identify deletions or rearrangements of one or several exons, which account for 16% of all aberrations in *RB1* [5].
- Cytogenetic studies such as karyotype or chromosomal microarray can be used to identify chromosome translocations or deletions.
- Sequence analysis of RNA from blood can help to identify splicing mutations that cannot be detected on routine DNA sequencing.
- *RB1* promoter hypermethylation analysis is used to detect silencing of *RB1* expression in tumor DNA. Hypermethylation of the promoter region occurs in 10–12% of nonheritable retinoblastoma [4].
- Linkage analysis can be utilized to track the mutant gene in families with two or more

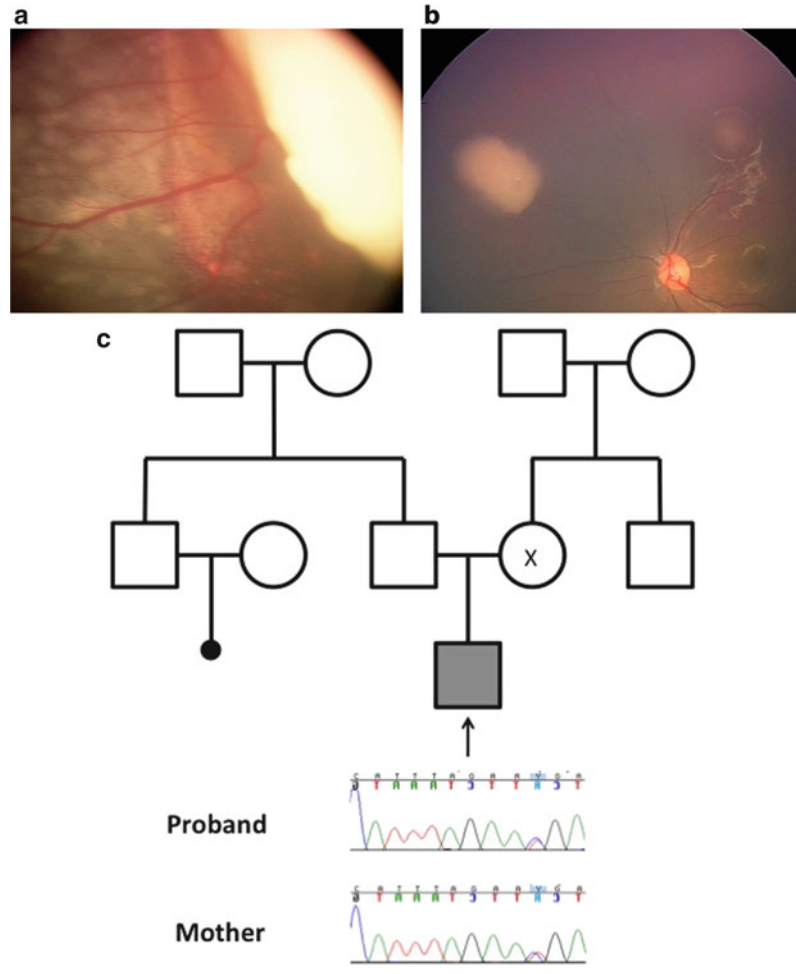

Fig. 17.2 A 3.5-year-old boy presented with multifocal bilateral retinoblastoma (**A**, right eye; **B**, left eye). A family history revealed that his mother had been enucleated in early childhood for "trauma" to the eye. Genetic testing (**C**) confirmed that the proband and his mother carried identical *RB1* gene mutation (c.1654C>T (p. Arg552X)

affected relatives when a mutation cannot be detected by conventional analyses. Polymorphic markers near the *RB1* locus are used to distinguish the normal allele from the mutant allele.

- Single-site mutation analysis is used to test at-risk relatives for the presence of the mutation already identified in the proband.

17.12 Genetic Counseling

The purpose of genetic counseling is to educate the family (and when older, the patients themselves) about the heritability of retinoblastoma, genetic testing, risks of future malignancies, recurrence risks for relatives, and reproductive options. All families of children with a diagnosis of retinoblastoma should be referred for a consultation with a genetic counselor or other qualified professional. Genetic counseling should occur soon after the initial diagnosis and again when a survivor enters reproductive age or prior to planning a family.

17.12.1 Initial Consultation

An initial consultation with the family in a genetics clinic will help provide a basic overview of retinoblastoma, especially its genetic intricacies.

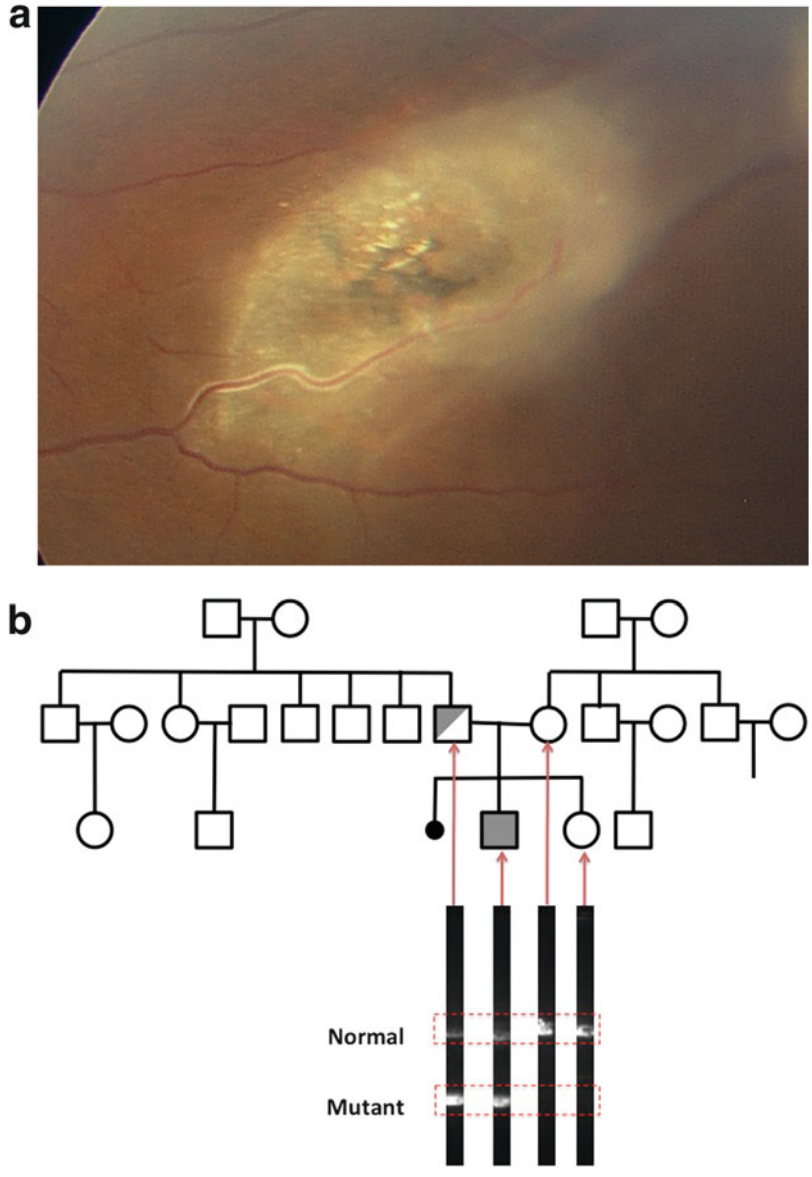

Fig. 17.3 A 2.5-year-old boy with bilateral retinoblastoma presented without family history of the disease. Retinal examination of his father revealed an asymptomatic retinoma (**A**). Genetic testing (**B**) for *RB1* gene mutation identified germline mutation in the proband and his father (g.170861_173866del [out-of-frame del of exon 25]). The sibling was unaffected by the mutation and therefore not subjected to serial examinations under anesthesia

The family members at risk can be identified when a family history has been obtained and a detailed pedigree drawn. The discussions would include the purposes of testing and likely outcomes of the test, test sensitivity, and residual risks. Genetic testing is then offered to the families, with their consent. Appropriate pretest counseling also help the parents understand the cost of testing. It is our experience that the cost of testing may vary based on the tests performed, with increasing costs when specimens are subjected to different technologies until a mutation is discovered or ruled out.

A second counseling session should be arranged to report and interpret the results. Once the results are reported, the family is advised to contact the relatives at risk so that they can be referred for counseling and testing.

17.12.2 Genetic Testing Outcomes

RB1 analysis is recommended in all children where results will influence surveillance or clarify recurrence risks for family members. The probability of a germline mutation varies based on tumor laterality and family history.

In unilateral retinoblastoma, tumor DNA is analyzed first to detect the two *RB1* mutations. Then, the proband's leukocyte DNA is analyzed for the presence of those mutations. If neither mutation is identified, the likelihood for heritable retinoblastoma is very low but cannot be ruled out due to the possibility of low-level mosaicism. In this case, the proband's potential future children should be tested for the mutations detected in tumor DNA, but no other family members necessitate genetic testing. If a tumor sample is not available and no mutation is identified in leukocyte DNA, the chance for an undetected germline mutation is approximately 1–1.5% depending on the laboratory's sensitivity. In children with unilateral retinoblastoma and negative genetic testing results, surveillance without anesthetic of the unaffected eye should occur until age 5 years. Offspring of such children may also receive surveillance without anesthetic [28].

In children with bilateral retinoblastoma, the germline mutation can be identified using only blood DNA in 90–95% of cases. When a germline mutation is unable to be identified, tumor DNA analysis may aid in recurrence risk assessment. If both mutations are identified in the tumor, but not in blood, low-level mosaicism caused by a postzygotic mutation in the proband is assumed, and only his/her future children would be at a low but definite risk to inherit the mutation. Other analyses to be considered when the germline mutation is not identified on sequence analysis include karyotyping to assess for chromosome translocations and RNA analysis to detect splicing variants. If two or more family members have retinoblastoma, linkage analysis may also be utilized to clarify the mutation status of at-risk family members. In some cases, loss of heterozygosity (LOH) testing in the tumor may be used to determine whether siblings and offspring are at risk. If LOH is detected, the haplotype analysis can be used to determine which allele carries the undetectable germline mutation. Siblings and children of the proband with the mutant haplotype are at increased risk for developing retinoblastoma and should be followed appropriately [29].

If genetic testing is not possible or is uninformative, empiric risk estimates can be utilized in genetic counseling. These risks are estimated from observational studies or calculated by multiplying the likelihood of a germline *RB1* mutation in the proband by the degree of relationship to the proband and likelihood of a mutation or mosaicism in the proband's parents [30].

17.12.3 Implications for Family Members

17.12.3.1 Children of the Proband

All future children of a proband with heritable retinoblastoma have a 50% chance of inheriting the *RB1* mutation. While the majority of children with heritable retinoblastoma have a de novo mutation, single-site testing for parents is recommended to clarify recurrence risks for siblings and extended relatives. In approximately 10% of cases, a parent has mosaicism for the mutation or may harbor the mutation and be unaffected [31]. The parent with a mosaic mutation is at elevated risk for second primary neoplasms and has up to 50% chances of transmitting the mutation to other offspring. If a child is diagnosed with a cytogenetic mutation such as a deletion or translocation, parents should also have karyotype analyses to determine whether one carries a balanced translocation. It is recommended that children with heritable retinoblastoma and *RB1* mutation carriers be monitored for new tumors with frequent examination under anesthesia or clinic visits until 5 years of age. Lifelong counseling for increased risk of other tumors is important [32].

17.12.3.2 Siblings

Risks of retinoblastoma for the proband's siblings depend on parental status. If a parent has a history of retinoblastoma, retinoma, or

positive genetic testing results, future offspring have a 50% risk of retinoblastoma. The risk may be lower for offspring of parents with mosaicism; but for genetic counseling purposes, a risk of up to 50% should be presumed. If neither parent's testing reveals the familial *RB1* mutation, there is still a small chance that one parent has germline, or gonadal, mosaicism that blood testing did not identify and subsequent children have a 2–3% chance of inheriting the mutation. Therefore, all siblings of a child with heritable retinoblastoma should be tested for the germline mutation. If the mutation is not identified, the sibling has the same risk of developing retinoblastoma as a child in the general population and does not necessitate surveillance. Any unaffected child found to have a germline *RB1* mutation should be examined under anesthesia every 3–4 weeks until the age of 1 and then every 3–4 months until the age of 5 years. Children with a known *RB1* mutation who receive frequent screening are diagnosed with retinoblastoma at an earlier age and have a better outcome than those who do not receive regular surveillance [33].

17.12.3.3 Parents

Studies have shown that the parents' perceived risk of retinoblastoma in their offspring strongly influences their decision to have children in the future [34]. When *RB1* germline mutation is detected, options for testing an embryo or fetus including pre-implantation genetic diagnosis (PGD) and prenatal diagnosis (PND) should be discussed with parents of an affected child as well as adult survivors of retinoblastoma. PGD is performed by testing an embryo for the presence of the *RB1* mutation following in vitro fertilization (IVF). It is important to note that de novo *RB1* mutations have occurred in children conceived by IVF [35]. Therefore, the small possibility of an unrelated *RB1* mutation, as well as unforeseen de novo mutations occurring throughout the genome during IVF, should be discussed. PND with chorionic villus sampling (at 10–12-weeks gestation) or amniocentesis (after 15 weeks gestation) may be used to determine whether a fetus is affected to either plan for surveillance or make decisions regarding continuing the pregnancy. These procedures are associated with a $\leq 2\%$ risk of miscarriage. If the mutation is identified prenatally, ultrasound can be used to identify some large intraocular tumors. Preterm delivery may be considered to allow for early treatment of tumors or early ocular examination [28]. If prenatal diagnosis is not performed, cord blood or an infant's peripheral blood may be used for testing after delivery. When test results are not yet available, at-risk children should have ocular evaluation soon after delivery.

It should be noted that mosaicism cannot be inherited, and therefore all antecedent relatives are cleared of risk. Future offspring of a mosaic proband still do require to be tested to determine if they have inherited the same RB1 mutant allele tumor as their parent's tumor.

17.13 Specific Issues in Asian Context

- Greater patient awareness about possibility of genetic transmission of retinoblastoma even in the absence of preexisting family history is required.
- Lack of realization that genetic counseling and genetic testing should be an integral component in the care of retinoblastoma children as well as their family members.
- Genetic testing is cost-effective as it identifies at-risk individuals requiring serial surveillance for onset of new intraocular tumors, trilateral retinoblastoma, and second malignant tumors. Unaffected individuals (*RB1* mutation absent) need not be subjected to intense surveillance.
- Accurate risk assessment and successful counseling allow families to consider reproductive options.
- Access to certified laboratories providing high-quality and reliable genetic testing for *RB1* is limited.

Compliance with Ethical Requirements

Ashwin Mallipatna, Meghan Marino, and Arun D. Singh 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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Molecular and Clinical Genetics of Retinoblastoma

18

Jason C.S. Yam, Winnie W.Y. Lau, Wai Kit Chu, Li Jia Chen, Kwong Wai Choy, Simon T.C. Ko, and Calvin C.P. Pang

Abstract

Retinoblastoma (RB) is the most common intraocular cancer in children affecting all populations. Prognosis is highly dependent on the timing of diagnosis. Late presentation with extraocular spread is often fatal. In contrast, early-staged diseases are mostly curable using systemic plus focal therapy. Therefore, early presymptomatic diagnosis and even prenatal diagnosis are crucial to improve the clinical outcome. In recent years extensive studies on the *RB1* gene and other RB-related genes have contributed to the better understanding of the disease mechanism. Inactivation of both copies of the *RB1* gene on chromosome 13q14, due to loss-of-function mutations, is a prerequisite for tumorigenesis in RB. Secondary genetic alterations, such as lesions in another gene, are necessary to precipitate tumour development. We have found in Hong Kong Chinese about 19% of RB patients carry a germ-line *RB1* mutation with no methylation at the *RB1* promoter. We detected inactivation of the *RB1* gene by loss-of-function mutations and loss of heterozygosity (LOH), but rarely by promoter hypermethylation, in Chinese sporadic RB. Promoter hypermethylation that silences gene expressions in the tumour suppressor gene *RASSF1A* and the DNA repair genes *MGMT* and *MLH1* is a causative factor of retinoblastoma. We have also revealed the presence of microsatellite instability and recurrent loss of heterozygosity at multiple chromosomal regions in the retinoblastoma genome, showing genes other than *RB1* as primary or secondary cause of

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retinoblastoma. Recently, we are working on extraocular regulation of RB development. Our studies on Chinese RB samples have led to useful information in the RB genome and cell biology for novel design of therapeutic modalities for RB.

Keywords
Retinoblastoma • *RB1* • RB genome • Diagnosis • Therapeutics

18.1 Introduction

Retinoblastoma (RB; OMIM 180200) is a cancer that originated from undifferentiated embryonic retinal cells in the developing retina in infancy or early childhood. It is the most common intraocular cancer in children worldwide [1]. Occurrence of RB is pan-ethnic, with clinical course and prognosis essentially similar in patients of different ethnicities [2]. About 40% of RB are hereditary and the rest are sporadic [3]. Most sporadic patients are unilateral and not transmissible with low risk of developing non-ocular cancer [3]. But some sporadic patients have bilateral tumours. A small proportion of them, whether unilateral or bilateral, may produce affected offspring. Patients with hereditary RB are predisposed to non-ocular cancer later in life. Their parents, siblings and children are susceptible to transmit or develop the disease [3, 4]. About 10% of hereditary RB present unilaterally, and some of these patients develop intra- and extraocular complications [5]. There are clinical implications whether a patient carries a germ-line (hereditary) or somatic (non-heritable) mutation. Germ-line mutations increase the risk of developing secondary tumours to 30% after 40 years and tertiary tumours to 22% [6].

Both hereditary and sporadic RB are caused by deleterious mutations in the *RB1* gene on human chromosome 13q14 [7], which is the first tumour suppressor gene identified. Transmission of RB at the cellular level is recessive [3]. Based on the statistical analysis of a large database, the genetics of retinoblastoma can be explained by a "two-hit hypothesis", which involves two mutational events or a doubly defective *RB1* locus [8]. Apart from chromosome 13, recurrent genetic alterations in RB include structural aberrations in 1q, 6p, 16 or 17 and regional loss on chromosome 19q13 or X [9]. In Hong Kong Chinese patients, we have shown that 19% of RB cases carried a germ-line *RB1* mutation [10]. We identified epigenetic silencing of DNA repairing genes (*MGMT* 35% and *MLH1* 67%) and a tumour suppressor gene *(RASSF1A* 82%) in RB [11–13]. Such epigenetic events through promoter hypermethylation in these three genes were mutually exclusive and together occurred at a high frequency of 86% in sporadic RB. Our genomic investigations of RB tissues in comparison with non-cancerous tissues of the same individuals also revealed microsatellite instability and recurrent loss of heterozygosity (LOH) at chromosomal regions of D19S902 and D22S539 in the RB genome [13]. On the cellular level, RB may be originated from naturally death-resistant retinal precursor cells or *RB1*-deficient retinoblasts undergoing apoptosis mediated by the p53 pathway [14]. We have found upregulation of a protein, B lymphoma Mo-MLV insertion region 1 (BMI-1), in RB tissues with high expression in undifferentiated cells with extraocular invasion. [15]. Studies on Chinese RB patients in Hong Kong thus revealed genomic information that is both pan-ethnic and ethnic-specific, including differential spectrum of mutations in the *RB1* gene and genetic architecture outside chromosome 13q14.

18.2 Epidemiology of Retinoblastoma: The Asian Perspective

Retinoblastoma is the most frequent ocular cancer of childhood, accounting for about 4% of all paediatric cancer. Globally, the incidence rates of retinoblastoma ranged approximately 40–60 per million live births worldwide, which corresponds to 1 per 15,000–25,000 live births. The disease has no validated geographic or population hotspots. The greatest burden is recorded in large populations that have high birth rates, such as in Asia and Africa [1]. Population-based studies have been conducted to document the incidence rates in Asia. Taiwan has reported an incidence of 1 per 21,691 live births during 1979–2003 [16] and 1 per 17,373 live births during 1998–2011 [17]. Singapore has reported an incidence of 1 in 15,789 live births [18] and incidence in Hong Kong is around 1 in 18,000 live births [19]. According to the Hong Kong registry from 1980–2009, there were 104 patients diagnosed with RB. Tables 18.1 summarizes the incidence rates in Asian regions [16–24].

There is no strong geographic difference in incidences by gender or laterality. However, studies from Mexico and Brazil have reported an inverse correlation between the incidence of RB and socioeconomic index [25, 26]. In more industrialized countries, an increased incidence has been associated with poverty and low levels of maternal education [27].

RB is mostly diagnosed before age five. Leukocoria, white reflex in the pupil, is the most common presentation in Hong Kong Chinese, accounting for 68% of cases [19]. It is usually first noted by the child's mother under dim illumination or flash photography. Another frequent initial sign of retinoblastoma is strabismus, accounting for 10% of cases. It usually correlates with macular involvement. Alternatively, children may present with poor vision, red eyes, anterior uveitis and hyphema. In contrast to the developed world where most cases are detected early, in developing countries the tumour is often detected in an enlarged eye with locally invasive disease. In Hong Kong, the survival rate is 95.1% during 1980–2009, i.e. five deaths among 104 patients. Survival rates in the United States approach 100% and are lower in many other parts of the world especially developing regions: 80–89% in Latin America [28], 83% in Iran [29], 81% in China [30, 31], 48% in India [32] and as low as 20–46% in Africa [33, 34]. As a result, 3000–4000 deaths annually have been estimated worldwide [1]. Notably, if survival rates worldwide approached those of Europe, the United States and Japan, deaths due to retinoblastoma could potentially be reduced by 88% annually, representing approximately 400 children globally [35].

18.3 Molecular Pathogenesis

RB arises from the cone precursor cells in the retina, a light-sensitive layer of cells of the inner part of an eye that conveys light into electrical signal and passes the signal to the brain

Table 18.1 Reported annual incidences of retinoblastoma in Asia

Region	Coverage	Source	Study period	Age <5 years per million	Live births
Japan	Nagasaki	Takano et al. [20]	1965–1986		one in 16,000
Japan	Nationwide	The Committee for the National Registry of Retinoblastoma [21]	1975–1985		one in 19,800
Singapore	Nationwide	Saw et al. [18]	1968–1995	11.1	
Vietnam	Ho Chi Minh	Nguyen et al. [24]	1995–1997	14.8	
Korea	Nationwide	Kim et al. [22]	1991–1993		one in 20,000
Korea	Nationwide	Park et al. [23]	1993–2010	11.2	one in 16,900
Hong Kong	Hong Kong Island	Yam et al. [19]	1996–2005		one in 18,000
Taiwan	Nationwide	Chen et al. [16]	1979–2003		one in 21,691
Taiwan	Nationwide	Li et al. [17]	1998–2011		one in 17,373

[36]. Loss of function of both copies of the *RB1* gene leads to the development of RB [14, 37, 38]. The gene product of *RB1* acts as a signal transducer connecting the cell cycle progression with the transcription machinery [39]. There are four stages in the life cycle of a cell: G1, S, G2 and cell division. Once the cells have divided, they will go into the G1 stage to start the next cell cycle again. In the G1 phase of the cell cycle, cyclin D is highly expressed, which leads to cyclin-dependent kinase (CDK) 4 and 6 activation [15]. CDK4 and CDK6 then phosphorylate RB, inhibiting RB binding to a transcription factor E2F [40, 41]. As a result, the RB-free E2F binds to promotors of several genes to turn on their expressions and induce cell cycle progression into the S phase, the DNA synthesis phase (Fig. 18.1). The premature progression into the S phase would trigger apoptosis, a programmed cell death to prevent uncontrolled cell proliferation [42]. However, the cone precursors reportedly expressed high levels of MDM2, a protein that suppresses p53-mediated apoptosis [36]. Therefore, cone cells in patients carrying *RB1* mutations pass through the cell cycle faster without triggering cell death. As a result, cone cells proliferate uncontrollably and turn into RB. Previously, we identified high expression of BMI-1 in RB from Chinese patients [15]. BMI-1 increased CHX10 expression but not nestin and neurofilament M, indicating association of BMI-1 with RB undifferentiation and progression. We also discovered BMI-1 induced the protein expression of cyclin D and to suppress apoptosis. As a result, BMI-1 promotes cell proliferation by speeding up the cell cycle progression and prevents these cells from dying. BMI-1 thus plays an important role in the development of RB [15].

18.3.1 RB1 and Genome Instability

As mentioned above, the premature progression into the S phase would trigger apoptosis [42]. In *RB1*-mutated cells, the RB-free E2F promotes the expressions of genes to induce cell cycle progression into the S phase. In these cells, the nucleotide levels may be too low to support the sudden increased levels of DNA replication in the S phase [43]. The restricted nucleotide pools would slow down DNA replication, resulting in unreplicated regions in the genome. Most of these unreplicated regions exist as DNA single-strand breaks (SSBs). However, if there are neighbour replication forks approaching the SSBs, the SSBs could be converted into DNA double-strand breaks (DSBs) [44]. In addition, following partial inhibition of DNA synthesis, some loci in the genome preferentially exhibit characteristics of DSBs, which are called "fragile sites" [45], which can be common or rare. Common fragile sites occur in all individuals, while

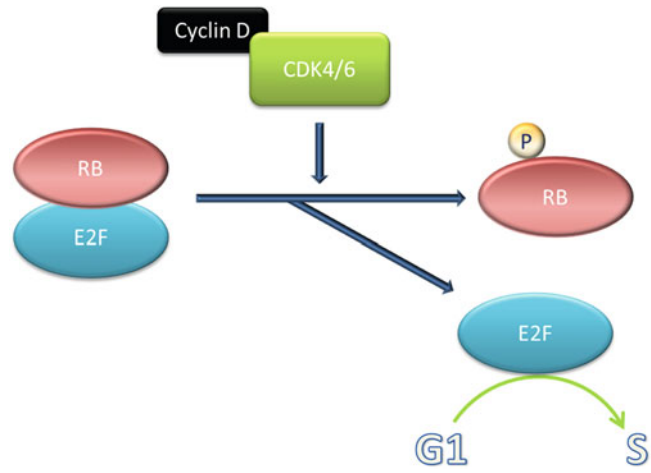

Fig. 18.1 In G1 phase, cyclin D is highly expressed and forms a complex with CDK4/CDK6. The cyclin D-CDK4/6 complex phosphorylates RB, which makes it dissociate from E2F. The free E2F in turn switches on the expressions of other genes to drive cells to progress into the S phase

rare fragile sites are found in a small proportion of people and are inherited in a Mendelian manner [45]. In cells with *RB1* mutation, cells would enter the S phase faster and have higher levels of DNA replication. However, the nucleotide levels are not enough to support high levels of DNA replication, which would potentially generate DSBs at fragile sites to become sources of genome instability and generate extra mutations such as gene deletions. Therefore, *RB1* can be considered as the primary mutation and the resulting DSBs secondary mutations.

There are two major pathways to repair DSBs: homologous recombination and non-homologous end joining. In the homologous recombination model, the DSB ends invade and copy a homologous sequence to repair the breakage sites [46]. In non-homologous end joining, the DSB ends are ligated together directly. Therefore, non-homologous end joining is considered to be "error-prone" as there could be some DNA sequences loss at the breakage sites. Homologous recombination is considered to be "error-free" as the homologous sequences are highly similar to the original sequences at the breakage sites [47]. On the other hand, the genome is considered to be "heterozygotic" as half of the genome was inherited from the mother and the other half from the father. As homologous recombination uses the homologous sequence to repair the damaged allele, the resulting alleles will carry the identical DNA sequences, and the original DNA sequence differences between these two alleles would be lost. Alternatively, non-homologous end joining may lose the DNA sequence from one allele, which causes cells to carry only one copy of the gene and thus lead to LOH. We have identified a new LOH locus at 13q31 (D13S265) with a high occurrence rate (67%) [10, 14].

On top of these two repair pathways, there are some surveillance pathways to detect the damaged DNA. SSBs and DSBs are detected by two protein kinases: ataxia telangiectasia and Rad3-related protein (ATR) and ataxia telangiectasia mutated (ATM), respectively [48]. Both ATR and ATM can trigger a series of phosphorylation cascades that arrest cells to prevent their progression into the next cell cycle or induce cell death when the DNA damages are too severe to be repaired. Indeed, ATR and ATM are the major kinases to phosphorylate p53 to induce senescence or apoptosis [49]. These surveillance pathways are considered as the "oncogene-induced senescence" model [50]. In this model, *RB1* mutation causes replication stresses, which are detected by ATR and ATM. The surveillance kinases then activate some downstream proteins including p53 to trigger senescence or apoptosis. However, in cone precursor cells, the intrinsic high levels of MDM2 inhibit p53 to suppress apoptosis [36]. These surviving cone precursor cells carry the damaged genome, which can proliferate and give rise to RB.

Apart from DSBs, the human genome is also under the thread of other forms of DNA damages. For example, during DNA replication, cytosine is usually incorporated into the newly synthesized DNA strand opposite to guanine. However, alkylation on the oxygen atom (O^6) of guanine would lead to incorrect incorporation of thymine opposite to the O-6-methylguanine, which generates a DNA mismatch mutation. O-6-Methylguanine-DNA methyltransferase (MGMT) is an enzyme that converts O-6-methylguanine back to guanine to prevent the mutation. Our previous work identified hypermethylation in the promotor region of MGMT, which leads to impaired expression of MGMT in RB [11, 12]. Therefore, accumulation of DNA mismatch mutations could further compromise the genome integrity in RB.

In addition to preventing DNA mismatch mutations, there is also a DNA mismatch repair pathway that can repair the existing DNA mismatch mutations. MutL homolog 1 (MLH1) is a key protein in this mismatch repair pathway to coordinate the sensing of the distorted DNA structure caused by the mismatch and the removal of the error-containing DNA region [51]. Subsequently, DNA polymerase would synthesize new DNA to repair the mismatch. Our earlier work identified hypermethylation in the promotor region of MLH1, which associates with null MLH1 protein expression in RB [13]. Again, this would lead to accumulation of DNA mismatch mutations and genome instability in RB.

18.3.2 Alterative Pathways Inducing Apoptosis in Retinoblastoma

Our current understanding about RB points to the inability to induce apoptosis to kill the genomic unstable cone precursor cells. Consequently, alterative pathways in inducing apoptosis should be involved. Growth hormone-releasing hormone (GHRH) is a hypothalamic hormone, which binds to the GHRH receptor (GHRH-R) and triggers the synthesis and secretion of downstream growth hormones (GH) [52]. Apart from the pituitary, the GHRH-GH pathway also functions in extra-hypothalamic normal and neoplastic peripheral tissues, mediated by insulin-like growth factor-1 (IGF1) [53]. Interestingly, apart from the pituitary, uvea, iris and ciliary body, our previous work also found that GHRH, GHRH-R and GH are highly expressed in the retina [54]. Indeed, GHRH-R antagonists have been shown to trigger apoptosis in various tumours including glioblastomas, endometrial cancer, prostate cancer and colon cancer [55–58]. There are multiple mechanisms of GHRH-R antagonist to induce apoptosis in these cancers, ranging from protein kinase C activation to cytosolic calcium ion elevation and DNA damage induction [56–58]. Although how these mechanisms lead to apoptosis are still not very clear, they could provide a potential MDM2-independent pathway to induce apoptosis in cone precursors, which could in turn induce apoptosis in RB.

18.4 Clinical Genetics

Hereditary RB is typically transmitted as an autosomal dominant disease with 80–90% penetrance [38, 59]. The disease-causing gene, *RB1*, containing 27 exons, is highly polymorphic, with mutations located in most of the coding exons. In the online *RB1* mutation database (http://rb1-lsdb.d-lohmann.de/home.php?select_db=RB1), over 1700 unique DNA variants on *RB1* have been reported. Mutations of *RB1* could be generated by somatic inactivation of both allele or with a germ-line mutation in one allele and a somatic inactivation of the second allele [10]. Several possible forms of genetic and epigenetic changes to the *RB1* gene have been implicated, including point mutations, small deletions, large structural changes and DNA hypermethylation at the promoter region [60]. Also, secondary genetic and epigenetic alterations in another gene(s) are required to precipitate tumour development [38].

The risk of family members of a proband with RB depends on whether or not the proband has a germ-line *RB1* mutation. All patients with familial, bilateral, or unilateral multifocal RB are usually carriers of *RB1* germ-line mutations. In addition, patients carrying a germ-line mutation bear an increased risk of developing secondary tumours [61]. In general, germ-line *RB1* mutations have nearly complete penetrance in hereditary RB (>99%) [62, 63]. In contrast, the detection rate of germ-line mutation in sporadic RB is lower. For example, in a Hong Kong Chinese RB cohort of 42 patients, eight of them (19%) carry germ-line mutations [10].

Though the *RB1* gene is highly polymorphic, a meta-analysis showed that nearly 40% of *RB1* mutations are recurrent and gather in 17 hotspots, comprising 12 nonsense, 3 splicing and 3 missense mutations; the remaining mutations are scattered along the gene, being most frequent in exons 9, 10, 14, 17, 18, 20 and 23 [64]. In a Caucasian RB cohort, nonsense and splicing mutations are the most frequent mutations in familial hereditary RB, accounting for approximately 50% and 26% of cases, respectively [65]. In a Hong Kong Chinese cohort of 42 RB patients, nonsense mutations occurred in about 26% of patients, and two nonsense mutations, E54X (exon 2) and R320X (exon 10), were recurrent, each identified in two patients [10]. In a Shanghai Chinese RB cohort (n=85), 36.5% of the patients carried null mutations, and R579X (exon 18) was detected in three patients [66]. Other recurrent nonsense mutations in *RB1* among Chinese RB populations include the R255X (exon 8) and R455X (exon 14) [67, 68]. These exons (2, 8, 10 and 14) are likely

mutation hotspots in *RB1* among the Chinese populations, although larger RB cohorts are needed for depicting a more detailed mutation profile.

In about 50% of RB children, however, the disease mechanism due to causative mutations remains unknown. Other genetic and epigenetic events could have occurred. For example, in Hong Kong Chinese, about 20% of sporadic RB patients carry a germ-line *RB1* mutation with no methylation at the *RB1* promoter [10]. In contrast, inactivation of the *RB1* gene by loss-of-function mutations and loss of heterozygosity, but not by promoter hypermethylation, accounts for a large proportion, about 60% of Chinese sporadic RB [10]. Genes outside the *RB1* locus are involved in RB pathogenesis: silenced expressions of *MGMT*, *MLH1* and *RASSF1A* due to hypermethylation in their promoters are direct causes [11–13]. There are also microsatellite instability and recurrent loss of heterozygosity at chromosomal regions of D13S265, D19S902, D20S117 and D21S1914 [9, 14]. Therefore, genetic counselling for RB should take into account genetic and epigenetic events along with *RB1* mutations.

RB can occur without *RB1* inactivation. A recent report estimated that around 2.7% of RB tumours carry no *RB1* inactivation [69]. These tumours expressed functional RB1 protein. Cone cell markers X-arrestin and CRX were highly expressed in these $RB1^{+/+}$ tumours, which suggest these $RB1^{+/+}$ tumours have the same cellular origin as the conventional $RB1^{-/-}$ tumours. However, some of these $RB1^{+/+}$ tumours showed *MYCN* oncogene amplification. Like RB1, MYCN has been reported to control the activation of E2F [70]. These findings suggest targeting MYCN might be an alternative treatment for RB.

18.5 Early Presymptomatic Diagnosis: Retinoblastoma Awareness Campaign

The most important objective of treatment for RB is to cure the disease and thereby preserve life and then, when possible, preserve the globe and vision [71]. The management of RB has evolved over the past two decades with improvements in clinical outcome and prognosis. Despite these advancements, children with extraocular RB are often fatal even with chemotherapy or radiation treatment. Although intraocular RB is usually curable, advanced stage intraocular RB always requires enucleation, which not only means a total loss of vision but also imposes cosmetic concern with the artificial eyeball replacement. Since RB presents within the first few years of infancy, alert of parents or family practitioners on early detection is crucial. Late detection of an affected child has a significant negative impact on patient's personality development and psychological maturation, with adverse effect on the whole family [72]. In contrast, if RB is diagnosed at its early stage, it can mostly be cured effectively with chemotherapy plus focal consolidative therapy with laser treatment, which can preserve the eyeball and useful vision with favourable results and minimal side effects [73].

18.5.1 Early Diagnosis to Improve the Outcome

Early detection and immediate referral of children with RB to trained ophthalmologists are the most fundamental steps to save lives and eyes [74]. Early diagnosis requires that parents and primary care providers recognize leukocoria, i.e. white pupil reflex, which is the most common initial sign [75]. Using the red reflex test to detect leukocoria is the fundamental basis of current paediatric screening practice for RB, which currently is the only paediatric cancer routinely screened in the general population of the United States [75]. The American Academy of Pediatrics and the American Academy of Ophthalmology suggest eye screening by primary care doctors or paediatricians for leukocoria at newborn to 3 months, 6 months–1 year, 3 years and 5 years [76, 77]. Despite this routine paediatric screening for leukocoria by medical professionals, more than 90% US children with

RB initially presented with leukocoria are detected by a parent [75]. In fact, parents can readily and reliably detect leukocoria using modern flash photography. Therefore, the most effective way to increase early detection is providing education to lay people since the time frame for observation is much greater for parents. Public education about leukocoria and RB is effective on the basis of leukocoria, which is an easy sign for lay people [78].

18.5.2 Global Trend of RB Awareness Campaign and the Success

There are many successful national campaigns worldwide for early diagnosis of RB, notably Brazil, Honduras and Mexico. In Brazil, a National Campaign for early diagnosis of RB was initiated in 2002. A video calling attention to leukocoria as a symptom of cancer on flash photography was broadcasted on several television channels throughout Brazil. A toll-free telephone number was available for more information. Educational materials were provided for the general public, primary healthcare workers and ophthalmologists and paediatricians. More than 100 cases had been diagnosed through the campaign from 2002 to 2008 [79]. In Honduras, the RB awareness campaign was initiated in 2003. Posters and flyers were distributed to parents during the annual vaccination campaigns in government health clinics. Education was provided to health-care workers. It was a very successful campaign, with an increase in number of patients referred to paediatric oncology, a decrease in lag time from 7.2 months to 5.5 months and a decrease in the proportion of extraocular cases from 73% to 35% [80]. In Mexico, a large public education campaign for the public, schools and health-care centres was developed. A National Retinoblastoma Registry was created to review RB management of different institutions and to develop the national protocol and continuous education program [28].

18.5.3 Hong Kong Current Situation

In Hong Kong, all children are screened at newborn and then at aged 5. The mean age of presentation of our cohort of RB patients are 10 months for bilateral cases and 26 months for unilateral cases. Therefore, none of them were detected by the two screening sessions, one at birth and one at 5 years old. Annual whole population screening program for RB in Hong Kong is not cost-effective. However, public awareness campaign to parents of the targeted group of children aged before 5 years and the advocate of parental self-screening would be the most cost-effective way to help improve the outcome of this group of children and families. Recently, we have launched the Hong Kong Retinoblastoma Awareness Campaign which will be linked to the city-wide vaccination program to promote parental awareness of the disease.

18.6 Treatment and Management

Significant changes have occurred in the treatment and management of RB in recent years. Often presenting with leukocoria and/or strabismus, if untreated, this paediatric intraocular malignancy can lead to extraocular invasion, metastasis and death [81]. The goal of treatment is first to save life, then preserve the globe and maximize vision. Treatment options and management strategies have become more complex and varied, depending on the age of presentation, unilateral or bilateral involvement, severity of intraocular RB (classified by the International Classification of Retinoblastoma, ICRB [71, 82, 83]), the presence of extraocular involvement, genetic and systemic status, parental preferences and treatment availability [84]. Regular examinations and treatment under anaesthesia with thorough ophthalmoscopy, fundus imaging, ultrasound and other investigative procedures are mandatory during active treatment of the condition. When the tumours are controlled, examinations, either under anaesthesia or in the

Table 18.2 Strategies and indications for treatment modalities for retinoblastoma[a]

Management of intraocular RB		Primary disease: treatment options	→	Secondary (residual or recurrent) disease; bilateral or unilateral involvement	Treatment options
Bilateral RB		IVC		RB	Local therapy
		IAC[b]			IVC
		Local therapy			IAC
		Enucleation			Periocular chemotherapy
Unilateral RB: ICRB group	**A**	Local therapy			Plaque RT[c]
					EBRT
					Enucleation
	B	IAC		Subretinal seeds	IAC
		IVC			IVC
		Local therapy			Periocular chemotherapy
	C	IAC			Plaque RT[c]
		IVC			EBRT
		Local therapy			Enucleation
	D	IAC		Vitreous seeds	IViT
		IVC			IAC
		Enucleation			(IVC)[d]
		Local therapy			Periocular chemotherapy
	E	Enucleation			Plaque RT[c]
		IAC			EBRT
		IVC			Enucleation
		Local therapy			

ICRB International Classification of Retinoblastoma, *IVC* Systemic intravenous chemotherapy, *IAC* Intraarterial chemotherapy, *Plaque RT* Plaque radiotherapy, *EBRT* External beam irradiation therapy, *IViT* Intravitreal chemotherapy

[a]These are general guidelines only; treatment strategy differs between cases; the management of RB often involves a combination of the above treatment modalities

[b]In selected cases, in some centres [5]

[c]Plaque radiotherapy is currently unavailable in Hong Kong

[d]Response of IVC for control of vitreous seeds is usually less effective

clinic when the child is cooperative, can be performed less frequently [85].

Enucleation has been performed for decreasing mortality due to RB and remains to be important for managing cases of advanced RB today [1]. In the 1980s, external beam radiotherapy (EBRT) was commonly performed as a means to save the globe, but later this modality was no longer the treatment of choice due to its risks of causing secondary malignancy [86]. Systemic intravenous chemotherapy (IVC) has been used for chemoreduction and globe-saving strategies in the management of RB [87]. The more recent development and use of intraarterial chemotherapy (IAC) [88–90] and intravitreal chemotherapy (IViT) [91, 92] have provided more options to safely save life, preserve the globe and maximize vision. Table 18.2 summarizes the strategies and indications for treatment modalities for retinoblastoma.

18.6.1 Local Therapy (Laser/Cryotherapy)

Local treatment of RB includes laser or cryotherapy [81] as primary treatment of small tumours (mainly ICRB group A) or during and after systemic chemoreduction or other treatment modalities. Diode laser is extensively used to deliver heat onto the tumour surface usually over multiple treatment sessions to ultimately

aim at achieving a flat or calcified scar. Argon diode (and in the past, xenon) laser may also be applied around the tumour to cut off its blood supply in order to induce regression. Cryotherapy may be used for small and more peripheral tumours, using a triple freeze-thaw technique.

18.6.2 Systemic Intravenous Chemotherapy (IVC)

In the 1990s, IVC gradually replaced EBRT in its role for the treatment of RB to save globes. Worldwide, a regimen consisting of three chemotherapeutic agents is commonly used, which includes carboplatin, etoposide and vincristine (VEC regimen) [87, 93]. It is given for six to nine consecutive cycles, every 3–4 weeks, with examinations under anaesthesia (EUA) after each cycle, and consolidating with local therapy where possible. Results according to the ICRB showed that globe save was achieved in 100% of group A eyes, 93% of group B, 90% of group C and 47% of group D [87]. Adjuvant systemic chemotherapy is also given if high-risk features (including post-laminar optic nerve involvement, massive choroidal invasion) are seen on histological examination after enucleation to possibly reduce the risk of systemic involvement [84, 94, 95]. IVC needs to be administered, and the child closely monitored by paediatric oncologists for side effects and potential systemic toxicity [93, 95].

18.6.3 Intraarterial Chemotherapy (IAC)

IAC has been increasingly used around the world. It was first reported as an alternative modality for globe conservation while minimizing risks of systemic toxicity [96]. IAC may be used as a primary treatment modality (more commonly in unilateral cases), as well as for salvage cases after other modalities of treatment, including IVC [97]. Chemotherapeutic agents used include melphalan, topotecan, carboplatin, or a combination regimen [88–90, 93]. IAC may be repeated according to the clinical situation [98]. In a proportion of RB patients, IAC has been well tolerated, resulting in high success rates and no reported deaths [88–90, 97].

18.6.4 Intravitreal Chemotherapy (IViT)

Melphalan has been reported to be an effective intravitreal chemotherapeutic agent for RB [99, 100]. The use of IViT for RB for the treatment of vitreous seeds is often more difficult to control in conventional methods such as IVC [101]. In one study, intravitreal injections of (20–30 μg) melphalan were used every 7–10 days, resulting in globe retention of 87% (20 of 23 eyes) [101]. The use of melphalan is popular in recent years based on successful reports for the treatment of vitreous seeds in RB [91, 92]. But there are concerns with potential tumour seeding through the needle track [102], and reports on retinal toxicity [103] cause cautions in the administration and dosing of this treatment.

18.6.5 Periocular Chemotherapy

Periocular chemotherapy is usually delivered via the subconjunctival or sub-tenon route, in order to augment the intraocular concentration of the therapeutic drug [104]. However, its use has been limited due to its complications and inability to cure RB when used alone [84, 93].

18.6.6 External Beam Radiotherapy (EBRT)

Some decades ago, EBRT gained popularity as an alternative to enucleation and a means to conserve the globe [105]. It is effective for treating both vitreous diseases and the RB tumour itself. However, the increased incidence of second cancers [86] and poor cosmetic outcomes has led to avoidance of EBRT where

possible, limiting its use as an adjunct or salvage modality to recurrent or residual disease.

18.6.7 Plaque Radiotherapy (Plaque RT)

Plaque RT was developed as a means to deliver radiation to the tumours while avoiding the dreaded risks of EBRT. Nowadays, it is reserved for selected cases, usually as an adjunct or as a secondary treatment for recurrent or residual disease [1]. Due to the limited indications in RB and in other types of ocular cancers, plaque RT is not currently available in Hong Kong.

18.6.8 Enucleation

Despite the increasing trend of globe-conserving modalities for the management of RB, enucleation remains to be a safe and effective way of treating advanced RB, with the important aim of saving life as well as avoiding the possible complications that may occur in other modalities of treatment [1, 84, 106–108]. This is particularly important in the developing regions when RB often presents late and other treatment options may not be readily available [84, 106, 108]. Histological examination of the enucleated eye is essential to identify any high-risk pathological features, which may indicate the need for adjuvant systemic IVC as described above [95, 109]. In the event that residual disease was detected on the cut end of the optic nerve, adjuvant systemic IVC and radiotherapy would also be indicated [84].

18.6.9 Extraocular and Metastatic Retinoblastoma

Despite recent treatment advances, delay in presentation and/or refusal of treatment may cause RB to extend beyond intraocular disease. Treatment may involve high-dose chemotherapy and radiotherapy with or without autologous haematopoietic stem-cell rescue of the bone marrow, surgical resection and radiotherapy of site-specific metastatic disease [84, 110]. Further details of the management of extraocular and metastatic RB are beyond the scope of this book.

18.6.10 Remarks on RB Management

The management of RB consists of a multidisciplinary approach by ophthalmologists, paediatric oncologists, radiologists, clinical geneticist and other specialized health-care workers, taking care of the child and family holistically. The management protocol has rapidly evolved over the past decade, with novel therapeutic modalities. While saving the child's life is of paramount importance, there has been evolution of new management strategies and remarkable progress in new treatment modalities, which allow RB to remain as one of the most curable paediatric cancers while continuing a trend in increasing the rates of globe conservation and improving vision.

18.7 Future Prospects

We now have a much clearer picture in the genomic architecture of RB than just a few years ago. Cellular studies will be advanced in the future to throw light on the detailed mechanism. Reliable and robust protocols are available for pre-implantation, prenatal and presymptomatic detection [60]. More information has been revealed in ethnic-related genotype-phenotype associations [111, 112]. It is gratifying that treatment has been much improved, and recovery rate can be as high as 90% in many regions of the world [84]. Earlier detection with appropriate treatment leads to a much better clinical outcome. However, care for RB patients has to extend to psychological and social support [113]. Hopefully, we shall keep up our good work to tackle RB.

Compliance with Ethical Requirements

Jason C.S. Yam, Winnie W. Y. Lau, Wai Kit Chu, Li Jia Chen, Kwong Wai Choy, Simon T.C. Ko and Calvin C.P. Pang 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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Current Research Perspectives in Understanding Diabetic Retinopathy

19

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Abstract

Diabetic retinopathy (DR), one of the severe microvascular complications of diabetes, could be sightthreatening when not intervened at the earlier stages of the disease. With the persistent increase in the global prevalence of diabetes, DR pose a major public health concern and has been indicated as the 5th most common cause of blindness in the world. The review details the clinical features observed in DR , its classification and the associated risk factors that makes a diabetes person susceptible for retinopathy changes. The reveiw provides an overiew of the various strategies namely, gene mapping, expression profiling and animal models studies for diabetic retinopathy.

Keywords

Diabetic retinopathy • Genetics • Animal models • Expression studies

19.1 Introduction: An Overview on Diabetic Retinopathy (DR)

19.1.1 Global Burden of Diabetes and Its Complications

Diabetes mellitus (DM) is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia [1, 2]. According to the International Diabetes Federation (IDF), diabetes affects 366 million people worldwide, and this number is expected to reach 552 million by the year 2030. This equates to approximately three new cases every ten seconds or almost ten millions per year [1, 3]. Type 1 DM (T1D) earlier referred as insulin-dependent diabetes or juvenile-onset diabetes [1] results from a cellular-mediated autoimmune destruction of

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the ß-cells of the pancreas causing reduction in insulin levels, while type 2 DM (T2D) results due to insulin resistance, referred as non-insulin-dependent diabetes or adult-onset diabetes [1]. Other forms of DM include maturity-onset diabetes of the young (MODY) and maternally inherited diabetes and deafness (MIDD) [4, 5].

T1D most often occurs in <10 years of age and in young adults (<18 years). Its incidence has been reported to be increasing in both rich and poor countries [6]. T2D, a serious global health problem throughout the world, constitutes 85 to 95% of all diabetes [7] due to rapid cultural and social changes, aging populations, increasing urbanization, dietary changes, reduced physical activity, and other unhealthy lifestyle and behavioral patterns [7]. The morbidity and mortality in diabetes arises due to the long-term complications and systemic dysfunction that decrease the life expectancy by an average of 5–10 years [8].

Diabetic microvascular complications affect small blood vessels resulting in renal failure (nephropathy), blindness (retinopathy), and distal extremity amputations (neuropathy) mediated by both glycemic and non-glycemic factors [8]. The systemic effect of diabetes on large blood vessels (referred as diabetic macrovascular complications) leads to coronary artery disease, peripheral arterial disease, and stroke [8].

It has been observed from the Diabetes Control and Complications Trial (DCCT), and the United Kingdom Prospective Diabetes Study (UKPDS), that glycemic index implicated by the level of glycosylated hemoglobin (HbA1c) correlates directly to the severity of diabetic complications in both types of diabetes. Lowering HbA1c from 8.0% to 7.2% reduced the microvascular complications from 34 to 76% in T1D patients. Additionally the non-glycemic factors including hypertension, smoking, and obesity have been shown to contribute to the risk of neuropathy, nephropathy, and cardiovascular diseases [8]. This indicates the cumulative effect of both glycemic and non-glycemic factors in diabetes and its complications and also demands a more holistic approach for disease prevention strategies [9].

19.1.2 Diabetic Retinopathy

Diabetic retinopathy (DR), one of the severe microvascular complications of diabetes, could be sight threatening when not intervened at the earlier stages of the disease [10]. With the persistent increase in the global prevalence of diabetes, DR poses a major public health concern and has been indicated as the fifth most common cause of blindness in the world, accounting for ~4.8% of global blindness [11] among adults in the working age group, worldwide [12].

19.1.3 Current Method of Classification of DR

DR has been classified based on phenotypically recognizable lesions on the retina. It is important to recognize three distinct phenotypes from clinical point of view, namely, no DR, non-sight-threatening DR, and sight-threatening DR [13]. Non-sight-threatening DR includes mild and moderate nonproliferative DR and represents reversible stages of DR and calls for a good control of metabolic factors [14]. Sight-threatening DR includes severe NPDR, diabetic macular edema, and PDR [15]. In an attempt to simplify the classification of DR, the International Clinical Disease Severity Scale for DR was created [16]. This disease severity scale is based upon the findings of the Wisconsin Epidemiology Study of Diabetic Retinopathy (WESDR) and the Early Treatment DR Study (ETDRS) [17, 18]. According to this, DR is classified into five stages: no apparent retinopathy, mild nonproliferative retinopathy, moderate NPDR, severe NPDR, and proliferative DR (Fig. 19.1). The same group has also classified diabetic macular edema (which can be present with any of the stages of retinopathy) as mild, moderate, and severe diabetic macular edema. However, recently with intravitreal anti-VEGF being the first-line therapy for macular edema, diabetic macular edema is clinically graded (Fig. 19.2) as center involving and center not involving [16].

The initial pathophysiology is the pericyte damage and basement membrane thickening, progressing to blood-retinal barrier breakdown

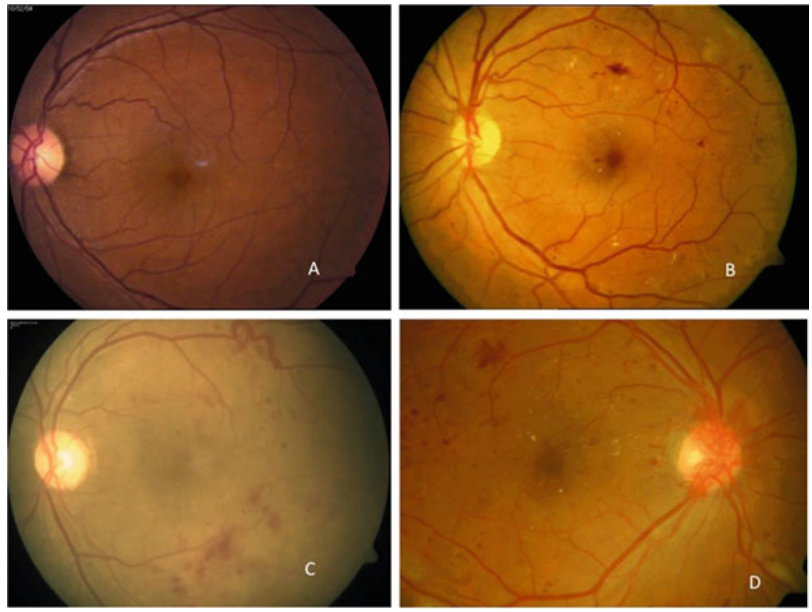

Fig. 19.1 Classifications of diabetic retinopathy: (**a**) mild nonproliferative, (**b**) moderate nonproliferative, (**c**) severe nonproliferative, and (**d**) proliferative diabetic retinopathy

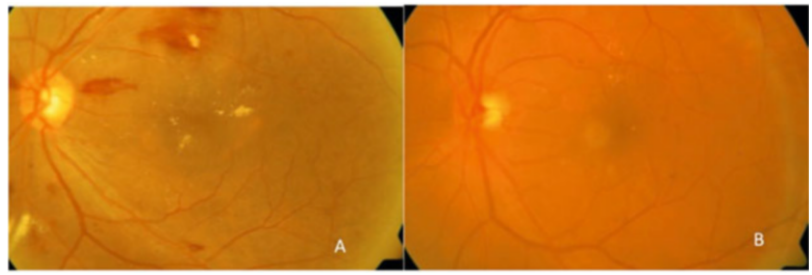

Fig. 19.2 Fundus of diabetic macular edema with (**a**) and without (**b**) involving the center of the retina

and increased permeability and then capillary closure, ischemia, and neovascularization [19]. There are multiple biochemical pathways which determine the pathophysiological process and the resulting phenotype [20, 21].

19.1.4 Functional Changes in Diabetes Without Recognizable Clinical Retinopathy

Functional tests may lead to additional useful measures of retinal changes prior to the onset or progression of DR. Several tests of visual function are abnormally early in DR, including decreased color vision, contrast sensitivity, and dark adaptation, even with normal visual acuity [22, 23]. Frequency doubling technology (FDT) response, which is indicative of ganglion cell function, may be a highly sensitive measure for discriminating between patients with and without early DR [22, 23]. Studies of multifocal electroretinography (mfERG) have also shown that delays in mfERG response can predict future sites of DR as well as predict progression to retinopathy within the next year [24, 25].

19.2 Risk Factors for DR

Epidemiological studies in many populations have identified clinical risk factors for

DR. Population-based studies from India [26, 27] have identified the following risk factors in Indian population. The most significant risk factor in both rural and urban India is the longer duration of diabetes (6.5 times more risk in people with >15 years duration of diabetes) [27, 28]. Two times risk for developing retinopathy (inclusive of sight-threatening retinopathy) is being observed in people developing diabetes with a younger age of onset (before 40 years) [29]. It has been reported that at the time of diagnosis, 1 in 10 persons had nephropathy and neuropathy and 1 in 20 have retinopathy [30]. Obesity increases the chances of developing DR. In Indian population, those with central obesity are associated with two times increased risk for DR [31].

The occurrence of DR is more in diabetics who take low fiber diet in comparison with people who take high fiber diet (20% vs 15%) [32]. People with suboptimal glycemic control (HbA1c>7) have more risk for having DR, and those with poor control (HbA1c >8) also had more risk of sight-threatening retinopathy [33]. Abnormal serum lipids (especially serum cholesterol and LDL cholesterol) have a major role in diabetic macular edema (both in center involving and center not involving diabetic macular edema) [34].

People with combination of suboptimal control (blood sugar, blood pressure, and lipids) are at higher risk for developing both retinopathy and sight-threatening retinopathy. Retinopathy changes are being reported in (i) nearly 1 in 3 diabetes people with suboptimal control of diabetes [35], (ii) two times more risk of retinopathy in early nephropathy (presence of microalbuminuria), (iii) six times more risk of retinopathy in advanced nephropathy (albuminuria) [36], and (iv) two times more risk with anemia [37].

Hyperglycemia The current diagnostic criteria for diabetes by the World Health Organization (WHO) and American Diabetes Association (ADA), based on the glycemic threshold, accurately separates persons at high and low risk of microvascular complications, specifically for the retinopathy signs of diabetes [38]. Poor glycemic control has been shown to cause chronic oxidative stress, a central mechanism for glucose toxicity [39].

Results from two landmark studies DCCT and UKPDS have shown significant benefit of intensive glycemic control (graded based on HbA1C levels) on the development and progression of DR [40]. The change in HbA1c was determined as independent risk factor for the development of DR and nephropathy by the Finnish Diabetic Nephropathy (FinnDiane) Study [40]. Similarly, the Wisconsin Epidemiology Study of Diabetic Retinopathy (WESDR) has shown reduction in incidence and progression of DR. The risk of retinopathy was shown to be lowered by 30–40% with every percent reduction in HbA1c levels [41, 42]. However, certain patients with poor glycemic control remain unaffected by the diabetic complications when compared to those with good glycemic control as observed in the DCCT trial [43]. The level of HbA1c level was strongly related with diabetic complications instead of the type of treatment [44].

Diabetes Duration The diabetes duration remains as another strong predictor for DR. The prevalence of DR is being reported as directly proportional to the duration of diabetes by many epidemiological studies (WESDR, DCCT). The incidence of developing PDR increased from 0% at 5 years to 27.9% at 14 years and remained stable after 15 years of diabetes. An increased prevalence of DR (87.5%) in patients with greater duration of diabetes (>15 years) when compared with the group which had <15-yr. duration (18.9%) was also observed. The Chennai Urban Rural Epidemiology Study (CURES) showed 41.8% of DR patients had > 15 years duration of diabetes, and for every 5-year increase in duration of diabetes, the risk for DR increased by 1.89 times [45].

Hypertension Hypertension exacerbates DR through increased blood flow and mechanical damage of vascular endothelial cells through the release of VEGF. Epidemiological studies (WESDR) and the United Kingdom Prospective

Diabetes Study (UKPDS) as well as clinical trials strongly support the role of hypertension as an important modifiable risk factor for DR. According to the UKPDS study, tight blood pressure control reduced the risk of DR progression by about a third, visual loss by half, and the need for laser treatment by a third in people with T2D [45].

Dyslipidemia An abnormal amount of lipid referred as dyslipidemia has been suggested to play major role in the pathogenesis of DR. The DCCT study has shown a proportional (increased triglyceride) and inverse (HDL cholesterol) correlation of the lipid parameters with the degree of DR severity. The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) trial has shown that fenofibrate, a lipid-modifying agent, reduced the need for laser treatment of STDR by 31% in patients with T2D. However this study does not report the effect of the drug on the change in lipid profile and the corresponding mechanism in reducing the risk for DR changes [46].

Genetics The prevalence of DR among diabetes patients irrespective of the glycemic index suggests the role of genetic factors in regulating DR complications. The results of few epidemiological studies and clinical trials have shown that the incidences of retinopathy were not altered even after intensive control of glycemia and blood pressure [47–49]. Microaneurysms were also observed in nondiabetic subjects with normal HbA1c level [50]. Additionally identical twins with diabetes showed same degree of retinopathy [51]. Also a recent study by Hietala et al has shown heritability for the severe manifestation of DR [52]. Thus these observations suggest the role of factors other than hyperglycemia or blood pressure in the development and progression of DR [53]. Population-based studies have also suggested an increased prevalence of DR-specific ethnic groups: African Americans, Hispanics, and south Asians in comparison with Caucasians. For example, results from the UK Asian Diabetes Study have shown that after controlling the risk factors of retinopathy, people with a south Asian ethnic origin showed high risk for DR than the Caucasians [46]. However no significant difference in the prevalence of DR has been found based on racial or ethnic difference among Chinese, Malays, and Indians living in Singapore [54]. The Multi-Ethnic Study of Atherosclerosis (MESA) reported moderate differences in the prevalence of DR among different races. Higher prevalence of DR reported in African Americans (36.7%) and Hispanics (37.4%) than in the white Americans (24.8%) [55]. Mexican Americans and African Americans have been reported to have higher prevalence and severe retinopathy when compared to non-Hispanic whites [56, 57].

DR Clusters in Families Epidemiological studies have shown many clinical factors to increase the risk for DR like diabetes duration, primarily intensive glycemic control [57]. It is however noteworthy that control on these major clinical risk factors did not show significant differences on the incidence or progression of DR (ADVANCE, DCCT trials). Clinical studies on human subjects revealed significant variation in the onset and severity of retinopathy [9–12]. The ADVANCE trial demonstrated that intensive glucose control to reduce glycosylated hemoglobin or lowering of blood pressure to near normal levels or control of blood glucose levels did not correlate proportionately with DR onset or progression. These observations corroborate the genetic influence on DR pathology and were further supported by familial aggregation studies and twin analysis in different populations. Studies in various populations (whites, Pima Indians, south Indians, Chinese, Mexican American) have shown a heritability estimates that ranged from 18 to 27% for any type of DR and 25 to 52% for PDR, suggesting that the severity of DR rather than the occurence correlated well with family history [68,70,72]. The influence of genetic factors in DR pathology is also observed through twin studies. The risk for siblings and relatives of diabetes patients to develop DR has been found to be as high as threefold when compared to the families without history of DR. The Family Investigation of Nephropathy and Diabetes-Eye

study, in which nearly half of the 236 diabetic patients are Mexican Americans, demonstrated that the heritability of any DR in this population of type 2 DM is as high as 24%.

The Medalist Study showed that significant numbers (40%) of diabetes patients did not show mild degree of microvascular complications regardless of their HbA1c levels. In such cases, other classical markers are thought to be important predictive markers for diabetic complications. These data suggest that they might possess endogenous protective factors that can neutralize the adverse effects of hyperglycemia [58]. Epigenetic factors are also important. The DCCT and Epidemiology of Diabetes Interventions and Complications (EDIC) studies reported that patients from the original DCCT study continue to have discordance in the development of microvascular complications, even 10 years after maintaining the same levels of glycemic control as shown by HbA1c. These findings showed that hyperglycemia might induce epigenetic changes that are not reversed easily [58]. Thus, diabetic complications are a result of interactions among systemic metabolic changes, such as hyperglycemia, differential local tissue responses to toxic metabolites of glucose metabolism, and genetic and epigenetic modulators.

Genetic Factors in DR Genetic factors contributing to the varying outcome of DR are identified both through hypothesis-driven and hypothesis-free approaches. Candidate genes with functional implications in the pathophysiology of DR are selected for under hypothesis-driven strategy. Since hyperglycemia, mediates the development of diabetic complications, thus mediated mechanisms are selected as potential candidates for genetic studies in DR. These include polyol pathway, DAG/activation of PKC pathway, increased oxidative stress, AGE formation and action, hexosamine pathway, renin-angiotensin system, vascular endothelial dysfunction, tissue matrix remodeling, and angiogenesis.

Candidate gene association studies in DR have been widely used across different populations. Polymorphisms in candidate genes of inflammation, oxidative stress, polyol pathway, growth factors, and other systems/pathways are studied for their association with DR in various populations including India. The various reports from Indian population are given in Table 19.1. Such studies are often inconclusive as most of these results are from small sample size and lack of replication studies and lost the p value of significance when corrected for multiple testing. Meta-analysis is also being attempted to overcome problems like sample sizes; however, the effect of ethnic differences/allele frequencies that could change the direction of association, publication bias, and clinical phenotyping differences across these studies

Table 19.1 List of candidate genes studied for association with DR in Indian population

Gene	Pathways	References
TNF	Inflammation	[60,65,67]
iNOS, eNOS,	Oxidative system	[59,65–67,71]
RAGE	Nonenzymatic protein glycation	[62,65,67]
AKR1B1	Polyol pathway	[64,65,67]
VEGF, IGF-1	Growth factors	[63,65,96]
PRKCB1	Influences other pathways such as inflammation, neovascularization, and aberration of hemodynamics	[61]
SERPINF1	Antiangiogenesis and neuroprotection	[61,65]
EPO	Hemopoeitic system/neuroprotection	[61]
CFH	Complement pathway	[61]
HTRA1	Regulates angiogenesis through GDF6	[65]
HFE	Hemochromatosis	[65]
ICAM-1	Adhesion molecules	[65,97]
Toll-like receptor 4	Immunity in the retina	[69]

poses a major problem to draw any conclusions on the association status. Genetic variants associated with DM and its other microvascular complications were studied for their association with DR (involving nine different cohorts and large number of SNPS (~49,000); however, no conclusive results were achieved except for SNPs in *SELP* gene [98] which again did not replicate suggesting screening of additional cohort.

19.2.1 Family Studies for Gene Mapping in DR

Genome-wide studies (GWAS) have been attempted in DR using both family-based-/case control-based strategies. There are a minimal number of linkage studies for DR (in Mexican Americans and Pima Indians) that indicated linkage on chromosome 1,3 and 12 for DR. [99,100] However these studies did not indicate any conclusive results as it is observed for linkage-based studies for Mendelian disorders. There are only limited number of linkage studies currently available for DR as these studies require large informative families (not possible for any complex diseases like DR due to the late onset of disease, heterogeneous clinical features), the low map resolution that offers only rough estimates of the genetic regions, and the cumulative effects of individual genetic variants with low effect size. It is being suggested that studies in inbred families using high-throughput technologies could augment the chance of gene identification in DR.

19.2.2 Genome-Wide Association Studies in DR

GWAS for DR are available for DR in Mexican American, Chinese, and white population with modest small size. Table 19.2 summarizes the GWAS available till date in various populations. GWAS studies in DR have identified many loci associated withDR. Huang et al. have identified five novel chromosomal regions and *PLXDC2* and *ARHGAP22* genes with susceptibility to DR [78]. These genes are implicated in endothelial cell angiogenesis and increased capillary permeability [78]. Genome-wide meta-analysis by Grassi et al. revealed an intergenic polymorphism rs476141 on chromosome 1 between genes, *AKT3* and *ZNF238*, associated with severe DR in different studies. Of these the *AKT3* regulates cell survival, insulin signaling, and angiogenesis after getting activated by both PDGF and IGF-1, both of which have been implicated in PDR. This analysis found an intronic SNP rs10521145 in *CCDC101*, a histone acetyltransferase, gene tagging a copy number region CNVR6685.1 on chromosome 16 by copy number variation (CNV) analysis [77]. However, none of the loci identified in these studies have been previously linked to DR or diabetes, thus suggesting the role of unsuspected pathways in the pathogenesis of DR [78]. A preliminary GWAS from the SN-DREAMS project based on 88 cases with sight-threatening DR (STDR) and 82 controls with duration of T2D $>$ 15 years, using the Affymetrix 250 K array,. The study identified did not reach genome-wide significance, and imputation was not done to boost the performance of the array. Our best results without imputation included several significant markers at a p value of 1×10^{-5} and less. These results suffer from low power due to the small sample size and the content and density of the array utilized for GWAS.

19.2.3 Gene Expression Studies in DR

DR is a microvascular complication and disruption of the integrity of the retinal vessels, the associated endothelial cells, and pericytes which have been well documented. Recently, visual dysfunction due to neuronal damage, especially rod photoreceptors, has been observed in NPDR cases [79, 80]. Many animal models on rat, mice, rabbit, dog, cat, swine, monkey, and zebrafish have been developed to understand the pathophysiological mechanisms of DR and its complications [81, 82]. However, animal model

Table 19.2 List of whole-genome association studies in DR

Ethnicity/population	Phenotype of cases	Genotyping platforms	Sample size(N		P value of significance	Putative candidate genes gene/function	Reference
			Cases	Controls			
Discovery: Australians; Replication: whites (T1D) and Indians (T2D)	STDR	HumanOmniExpress BeadChip	336	508	rs989605; 4.15×10^{-8}	GRB2 and MIR3678	Burdon et al. 75
Japan	PDR	Affymetrix	205(st1)	241	1.4×10–7	*RP1-90 L14.1*	Awata et al. [73]
		GeneChip 6.0	335 (st2)	288			
			297 (st3)	620			
China	PDR (type 1)	Whole-genome OmniExpress BeadChip, Illumina	570	437	rs9565164, $P = 1.3 \times 10^{-7}$	*TBC1D4-COMMD6-UCHL3*	Sheu et al. [74]
					(rs1399634, $P = 2.0 \times 10^{-6}$	*LRP2-BBS5*	
					rs2380261, $P = 2.1 \times 10^{-6}$	*ARL4C-SH3BP4*	
Caucasians (GoKinD and EDIC cohorts)	DME or DR (T2D; irrespective of DN status)	Affymetrix 5.0 platform	973	1856	rs476141, $P= 1.2 \times 10^{-7}$	*AKT3* and *ZNF238*	Grassi MA et al. [76]
	DME or PDR (T2D; excluding DN status)		281	1715	rs227455, $P= 1.6 \times 10–7$	rs227455 has been associated with expression levels of other genes (eQTL)	
	CNV analysis on DME or PDR T2D;excluding DN status)		281	1715	CNVR6685.1.	*SULT1A1* and *SULT1A2*,*In LD with CCDC101* and *NUPR1*	
Taiwanese	NPDR+PDR	Illumina HumanHap550-Duo BeadChip	174	575/100	P= $< 10^{-6}$	*MYSM1, FSTL5, C5orfF21, PLXD2, ARHGAP22*, and *HS6ST3*	Huang YC et al. [77]
Mexican Americans	NPDR+PDR (T2D)	Affymetrix GeneChip 100 K array	103	183	$P = 6.04 \times 10–5$	CAMK4	Fu et al.[78]
					$P = 6.21 \times 10–5$	FMN1	

studies have inherent limitations as many phenotype variations as seen in humans like retinal neovascularization and other exact human pathology cannot be mimicked [83]. Hence studying the disease tissue from humans helps in better understanding of the disease processes and outcome. Extensive studies on human donor tissue and vitreous samples from patients at various stages of DR have identified the primary pathology like (i) pericyte/endothelial cell death, (ii) neo-angiogenesis indicated by increased VEGF levels, (iii) inflammation indicated with increased cytokine level and adhesion molecules like ICAM, (iv) neuronal cell death suggested by fragmentation of ganglion cell nuclei, (v) and increased levels of pro-apoptotic proteins like Bax. Most of these findings have been confirmed on animal models or were as a lead from animal studies [79]. Gene expression analysis in vitreous samples and cultured endothelial progenitor cells of PDR cases has shown altered levels of markers for angiogenesis/inflammation. Further, miRNA profiling of vitreous samples has identified miRNAs involved in regulation of angiogenesis to be upregulated in PDR [84–87]. Owing to the difficulty in obtaining enough retinal tissue from the patients, sufficient enough to perform global gene expression profiling, there has not been much report on expression profile of whole transcriptome from DR cases. However, recently with the advancement of technology, global gene expression profiling of the fibrovascular membranes (FVM) from PDR cases identified 625 nonredundant ESTs including 110 novel ESTs. These were categorized by functional subsets of genes related to ribosomal activity, oxidative phosphorylation, focal adhesion, cell adhesion, and other functions [88]. Following this, it was identified and validated by vitreous expression studies in PDR cases that M2 polarization of macrophages from monocytes is induced by M-CSF and IL-13 in diabetic retinas and induced FVM formation through periostin production [89]. Thus global gene expression profiling provides enormous insight into differential expression in the disease condition which can further be taken for validation as biomarker or for designing drug therapies.

19.2.4 Animal Models of DR

There are a number of animal models for DR that have been validated for studying different aspects of pathophysiology of human DR. Diabetes can be induced in the animal models by different methods like streptozotocin (STZ) or alloxan injection, fructose or galactose diet, genetic manipulation, pancreatectomy. A number of spontaneously diabetic rodent models also are available for experimentation. Although significant knowledge on the etiology of DR has been gained from these animals models, unfortunately none of them can entirely replicate the human pathology. Most animal models represent only the early signs of DR, while some show the late-stage pathologic angiogenesis and vascular changes. However, to consider an animal model of DR, the following fundamental features should be present: (a) it should reflect structural abnormalities such as microaneurysm and accompanied by hyperpermeability, (b) easy maintenance of animals, (c) easy induction of diabetic or pathologic condition, and (d) validated methods of data interpretation [90]. The available animal models can be categorized as chemically induced diabetic models, spontaneously diabetic and proliferative retinopathy models in zebrafish, rodent, canine, and nonhuman primates. It is important to select a pertinent DR model for studying specific feature of DR.

Rodents Owing to the small size, easy handling, inexpensive maintenance, amenability to genetic manipulation, and similarity to human genetic background, the rodent models (mice and rats) have been widely used to study the molecular mechanisms underlying the pathogenesis of DR. In mice, either single dose of intraperitonial injection of 150–200 mg/kg STZ can induce hyperglycemia in 3 days–6 weeks or multiple doses of 40–80 mg/kg for consecutive 3–5 days can develop hyperglycemia in a week's time. However, according to the Animal Models of

Diabetic Complications Consortium, a standard protocol of 150 mg/kg single dose followed by multiple doses of 50 mg/kg for 5 consecutive days without insulin compensation has been recommended for inducing hyperglycemia in mice. Alloxan-induced hyperglycemic mice models are less commonly used as the cellular and vascular lesions were not clearly observed in the animals although functional abnormalities were reported. Galactose diet-induced DR mice models require longer periods to develop retinopathy. In addition to chemically modified and diet-induced DR models, spontaneous hyperglycemic mice due to endogenous mutation such as *db*/*db* mice, nonobese diabetic (NOD) mice, and Akita ($Ins2^{Akita}$) mice also serve as a good source for studying the mechanisms underlying DR. The broad range of transgenic and knockout mice expanded the knowledge on the pathological progression of DR recently. For instance, the role of endothelial nitric oxide synthase dysfunction in DR has been studied by inducing diabetes in eNOS knockout mice ($eNOS^{-/-}$) [91]. As the DR mice models do not exhibit the late-stage features of DR, nondiabetic mice with proliferative retinopathy are used to study the mechanisms of neovascularization. Akimba transgenic mouse model of DR has been developed by crossing the $VEGF_{165}$ overexpressing transgenic mice (Kimba mice) with spontaneous hyperglycemic mice (Akita mice) [92]. Akimba mice demonstrated extensive retinal neovascularization, increased vascular leakage, microaneurysms, venous beading, and attenuation of vessels. This model might be suitable for exploring the role of growth factor in the progression of DR.

Rat models of DR provide the advantage of larger tissue size for reproducible functional and molecular analyses compared to mice models. Similar to mice, STZ or alloxan or galactose diet can induce hyperglycemia in rats although with a much lower dosage as rats are more vulnerable to the toxicity of STZ. A wide range of STZ doses have been used to induce hyperglycemia in different strain backgrounds; for instance, a single dose from 30 mg/kg to 80 mg/kg was used in Wistar rats, Sprague-Dawley rats, Brown Norway rats, and Lewis rats. Depending on the genetic background, the onset of early symptoms of DR varied. However, the commonly used method is to inject a single dose of 60–65 mg/kg of body weight STZ. The chemically induced diabetic rats showed physiological and biochemical changes such as breakdown of BRB, increased VEGF, and neuronal damage after 1–-2 months of onset of diabetes. However, the retinal neovascularization was not observed in these animals. There are several spontaneous type 2 diabetic rat models such as Zucker diabetic fatty (ZDF) rats, WBN/Kob rats, Otsuka Long-Evans Tokushima fatty (OLETF) rats, Goto-Kakizaki rats, and Spontaneously Diabetic Torii (SDT) rats. Some of these models showed the late-stage DR features like pericyte loss, basement membrane thickening, focal nodules, and retinal vascular changes. Spontaneous type 1 diabetes model named biobreeding (BB) rats also demonstrate retinal changes after 8–11 months of diabetes.

Canine Canine animal models particularly dogs have several advantages over mice and rats as the structure of retina and size of the eyeball are similar to human. More importantly, the presence of macula in dogs which is absent in rodent and zebrafish models makes it a better system for the studies of DR. In addition, examination of the retinal structure and function can be easily accomplished by the clinically used instruments such as OCT and ERG. Spontaneously induced or STZ-induced or galactose-fed diabetic dogs showed retinopathy changes similar to human DR. However, galactose-fed dogs required longer feeding periods (more than 2 years), and the retinopathy changes can be observed between 3 and 5 years. In spite of the advantages, dog models of DR are practically less realistic due to difficulty in maintenance, shortage of molecular tools, strict ethical concerns, and higher cost. Next to dogs, type I diabetic cats induced by STZ and partial pancreatectomy with or without injection of alloxan reflect the pathological changes of human DR. As diabetic cats show

mild cataract, the fundus can be easily studied for longer periods after the onset of diabetes.

Diabetic Pigs The anatomy of retinal vasculature and size of the pig eye resemble that of humans. However, there are no fovea and central non-capillary areas in pig eye. Pigs develop diabetes with intravenous injection of 50 mg/kg STZ over 3 consecutive days, and the DR changes have been observed in 18 weeks [93]. Type 1 diabetic pig has been reported to demonstrate the pathological features of late-stage progressive DR, reasonably faster. The shortcomings of pig DR models are lack of specific antibodies for molecular studies, higher cost, and difficulty in maintenance although they would be important for validating the new therapeutic strategies prior to clinical trials in humans.

Non Human Primate Models of DM The presence of macula in primates besides the higher structural similarity to human makes them the prospective animal model for studying pathological events in DR. Rhesus monkeys develop diabetes with alloxan, STZ, or pancreatectomy. Spontaneous type 2 diabetes occurs in obese rhesus monkeys and cynomolgus monkeys. As in human, the DR changes in diabetic monkeys happen very slowly. Although the diabetic monkeys showed retinal ischemia, microaneurysms, and compromise of BRB, neuronal degeneration and vascular lesions were not observed even after 15 years of onset of diabetes [94]. The nonhuman primate models are not preferred over other models as the ethical concern is very high, there is slow progression of DR, and genetic manipulation cannot be done easily.

Zebrafish DR changes such as thinning of the inner plexiform and inner nuclear layers [95] and degeneration of photoreceptors and changes in cone-mediated ERG and increased thickness of BM in retinal capillaries indicating the compromise of inner BRB were observed in zebrafish DR model [96]. Hyperglycemia in zebrafish could be achieved by immersing the fish on alternative days in 2% glucose solution for a period of 30 days. Chemically induced diabetes in zebrafish was achieved by intraperitoneal or direct caudal fin injection of 350 mg/kg STZ. Vascular abnormalities can be effectively studied in the transgenic fluorescent zebrafish (fli1-EGFP-Tg) where the vascular endothelium is fluorescent labeled, aiding easy visualization and imaging of the blood vessels. Zebrafish is a very good DR model for understanding the cellular mechanisms and high-throughput drug screening, due to cost-effectiveness, minimal ethical concerns, and short life span. However, there are certain obvious disadvantages. The body structure, living conditions, the manner of reproduction, the retinal anatomy, and vasculature in zebrafish profoundly differ from humans. The requirement for skillful technique because of small size of tissue and lack of reagents for molecular studies also limit the usage of zebrafish as a model for DR [90].

As each diabetic animal model demonstrates unique features, the appropriate animal model should be selected based on the objective and duration of the study, maintenance cost, and ethical issues.

19.2.5 Future Perspectives

The different approaches attempted till date are not comprehensive enough to unravel the genetic architecture of DR due to the complex interplay of genetic and other systemic factors in DR. Alternatively, an integrated approach involving the genomic/proteomic/metabolomic signatures can impart the important knowledge on DR pathology that can eventually lead us to the identification of DR genes. The advent of next-generation sequencing technologies for whole genome/exome can offer a major breakthrough in DR research. These approaches can help in identifying the underlying copy number variations/rare variants. The role of CNVs in diabetic complications has been earlier implicated in type 2 diabetes-associated end-stage renal disease in African Americans and also in DR. Emerging

massive parallel sequencing technologies offer the platform to address the role of these rare genetic variants in DR.

These high-throughput studies demand the establishment of large colloborative efforts/consortia that could also address the other limitations for genetic studies in DR, namely, sample size, variations in the protocol for clinical phenotyping, availability of newer statistical approaches, and ethnic differences. An integrated approach, in addition to these, will eventually help in better understanding of the disease.

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Genome-Wide Association Studies of Glaucoma

20

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Abstract

Glaucoma is a common cause of blindness worldwide. The adult-onset glaucomas, including primary open-angle glaucoma (POAG), normal tension glaucoma (NTG), primary angle-closure glaucoma (PACG), and exfoliation glaucoma (XFG), are complex disorders with significant genetic contributions. Genetic risk variants influencing glaucoma disease have been identified using genome-wide association studies (GWAS). The review will summarize progress identifying risk factors for POAG, NTG, PACG and XFS and the related endophenotypes, intraocular pressure (IOP), optic nerve parameters and central corneal thickness (CCT).

Keywords

Genetic risk factors • Genome-wide association studies • Glaucoma

20.1 Introduction

Glaucoma is a group of multifactorial, slowly progressive, degenerative optic neuropathies, affecting more than 64 million people worldwide and is expected to increase to 76.0 million by 2020 [1]. The disease is characterized by the death of retinal ganglion cells (RGCs) and their axons and by associated morphological changes within the optic nerve and retinal nerve fiber layer (RNFL). Without treatment, the disease can progress to disability and irreversible loss of visual function.

The adult-onset glaucomas, including primary open-angle glaucoma (POAG), normal tension glaucoma (NTG), primary angle-closure glaucoma (PACG), and exfoliation glaucoma (XFG), are complex disorders with strong genetic components. Family history of glaucoma is a major risk factor [2–10]. For POAG, compared with the general population, first-degree

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relatives of affected individuals have a tenfold increase in disease incidence, and the risk of disease increases as the number of relatives with POAG increases [8, 11–14]. Glaucoma prevalence also varies among different ethnic groups and geographic ancestries [15, 16]. For example, Asians in general are at higher risk of primary angle-closure glaucoma (PACG), and those of Japanese descent are more likely to develop normal tension glaucoma (NTG). Interestingly, despite having a lower glaucoma prevalence, Asia has the largest absolute number of glaucoma cases worldwide, accounting for more than 60 % of the world population [1].

Significant progress in understanding the genetic architecture of glaucoma has been made over the last two decades. The etiology of glaucoma is complex and includes multiple genetic and environmental factors that individually have relatively small effects. Genome-wide association studies (GWASs) using cases and controls offer an unbiased approach to identify genetic variants associated with complex phenotypes. This approach has identified disease-associated SNPs in many chronic, age-related disorders with complex inheritance, including diabetes, heart disease, cancer, and others [17, 18]. GWASs require large numbers of unrelated cases and controls with sufficient power to identify statistical associations between single genetic variants (typically SNPs) and disease [19–23]. To adequately cover the genome, sometimes more than a million SNPs are tested for association with a disease phenotype or other trait. Given the many statistical tests performed, a "multiple testing correction threshold" for significance is generally set at 5E-8 (rather than $P<0.05$) after adjusting for correlated SNPs [24]. Replication of top signals is performed in independent cohorts to verify findings and reduce false-positive findings [25].

Smaller GWASs usually lack power to identify a signal with a modest genetic effect as detection of signals requires large sample sizes [26–29]. Consequently, meta-analysis is now a popular method for the discovery of new disease-related genes by increasing power and reducing false positives [30]. With the statistical synthesis of multiple independent GWASs, recent large-scale meta-analyses have discovered many novel associations. It is important to remember, however, that most of the associated SNPs are common and many unaffected people carry risk alleles, and most associated SNPs have small effect sizes ($OR<2.0$). Achieving the small P values that imply statistical association even after correction for the large number of SNPs tested in a genome-wide study provides support that the gene/loci are truly contributing to the disease or trait [29, 30]. To date, numerous glaucoma GWASs have identified important loci associated with POAG, primary angle-closure glaucoma (PACG), exfoliation syndrome (XFS) glaucoma, and numerous quantitative traits in glaucoma. These studies are the focus of this review.

20.2 GWASs and Quantitative Traits in Glaucoma

POAG and PACG are multifactorial and late-onset disorders that likely result from the complex interaction of both genetic and environmental factors. One approach to identifying the genetic components of complex diseases is to focus on underlying endophenotypes, which are typically measurable quantitative traits closely related to the disease phenotype [31]. Endophenotypes, also known as intermediate phenotypes, can be highly heritable, and the loci associated with the endophenotype could also predispose to the overall disease risk [32]. The endophenotype strategy for GWAS allows individuals to be ranked along the continuum of risk [33, 34] which can provide more power to detect statistical association compared to discrete categorical groups. Endophenotypes for POAG include cup-disc ratio (CDR) and vertical cup-disc ratio (vCDR), central corneal thickness (CCT), intraocular pressure (IOP), cup area (CA), and disc area (DA). Anterior chamber depth (ACD) is a PACG endophenotype. The heritability of these quantitative endophenotype is very high [3, 35–37].

20.3 IOP

In addition to family history and ethnicity, well-recognized risk factors for POAG include elevated IOP, age, myopia, and gender. Of these, elevated IOP is the most important risk factor in the development of POAG. It is also the only modifiable risk factor; and reduction of IOP decreases the progression of the disease in glaucoma cases with and without elevated IOPs [2, 38–41]. As a measurable endophenotype and important risk factor, IOP is an ideal target for GWASs.

GWASs have identified multiple loci associated with IOP, including *ARHGEF12* (11q23.3) [42], *TMCO1* (1q24.1), and *GAS7* (17p13.1) [43–45], which are highly expressed in the ciliary body, trabecular meshwork, lamina cribrosa, optic nerve, and retina. SNPs in the genomic regions for *ABCA1*, *FNDC3B*, *ABO*, and a three mega base pair region at 11p11.2 have been associated with IOP in Caucasian and Asian subjects [46]. *ABCA1*, one locus associated with high IOP in Asians [47], was found to be upregulated in mouse models of high-IOP glaucoma [47]. *ABCA1* encodes for a membrane-bound protein involved in phospholipid and cholesterol efflux [48, 49]. It is expressed in the cornea, iris, optic nerve, and trabecular meshwork and is highly expressed in the human retina and RGCs [46, 47]. Importantly, *TMCO1*, *ABCA1*, *FNDC3B*, and *GAS7* have also been associated with POAG in case/control GWASs [43, 45–47, 50].

20.4 Optic Nerve Parameters

Many loci for quantitative optic nerve endophenotypes have been identified. Many of these loci are associated with more than one optic nerve parameter: *CDC7*/*TGFβR3* and *CARD10* are associated with disc area (DA) and vertical cup-to-disc ratio (vCDR); *CDKN2B*/*CDKN2B-AS1*, *CHEK2*, *HSF2*, *COL8A1*, *SSSCA1*, *SIX1*/*SIX6*, *BMP2*, and *RERE* are associated with cup area (CA) and vCDR; and *ATOH7*, *SALL1*, and *TMTC2* are associated with DA, CA, and vCDR [51–53]. Although CA and vCDR are related, there are some CA loci not significantly associated with CDR or vCDR, including *ASB7*, *BCAS3*, *DCLK1*, *DDHD1*/*BMP4*, *DHRS3*, *EFEMP1*, *FAM101*, *FLNB*, *KPNB1*, *TRIB2*, and *TRIOBP30* [54], but this could be explained by lack of power in these studies. For DA alone, variants at *ABI3BP*, *CDC42BPA*, *DCAF4L2*, *F5*, *DIRC3*, *ELP4*, *HORMAD2*, *NR2F2*, and *RARB* have been identified [52, 54, 55].

Multiple GWASs have identified loci associated with more than one optic disc parameter and also POAG. One of the first genes reaching genome-wide significance in GWASs is cyclin-dependent kinase inhibitor 2B (*CDKN2B*), which is associated with both optic disc endophenotypes (CA and CDR) and POAG [24, 36, 43, 52, 56–68]. The "C" allele of rs1063192, in the *CDKN2B* genomic region, is associated with a decreased risk for POAG in a black Caribbean population and with decreased vCDR, while the "T" allele has been associated with increased vCDR and NTG in other populations including Caucasians [43, 52, 56–61]. *CDKN2B* is adjacent to *CDKN2A*, which is another tumor suppressor gene on chromosome 9p21 in a region that is often mutated or deleted in a wide variety of cancers. Both genes encode a cyclin-dependent kinase inhibitor (p15), which forms a complex with CDK4 or CDK6 and prevents the activation of the CDK kinases. The encoded protein from this locus induces G1-phase cell cycle arrest and limits cell growth. Therefore, *CDKN2B* upregulation is associated with cell cycle arrest which could be related to glaucoma development [62].

CDKN2B-AS1, or *CDKN2B* antisense noncoding RNA 1, is located in the same genomic region as *CDKN2B* and *CDKN2A* but in the antisense direction. *CDKN2B-AS1* SNPs have been associated with multiple diseases. Elevated levels of *CDKN2B-AS1* suppress the transcription of both *CDKN2A* and *CDKN2B* [63, 64]. This locus is significantly associated with POAG in Caucasian, Indian, and Japanese populations [43, 56–60, 65–68]. Additionally, association between this locus and POAG/NTG

has been identified in black populations in the Caribbean and Ghana [61, 69].

Expression of *CDKN2B* was found to be dramatically induced by transforming growth factor beta (TGF-β) [62]. TGF-β is a multifunctional peptide and cytokine that controls cell proliferation, differentiation, adhesion, migration, and other functions in a wide variety of cell types. TGF-β signaling has been implicated in a wide variety of diseases including inflammation, autoimmune disorders, fibrosis, cancer, cataracts, as well as POAG [62, 70–72]. TGF-β inhibits the progression of the cell cycle and is associated with programmed cell death in the developing optic nerve, RGC death, and glaucomatous optic nerve damage and may play important role in glaucoma [70, 72–76]. Burdon and colleagues suggested that increased IOP may increase expression of *CDKN2A* and *CDKN2B* [43]. These data may suggest a link between the *CDKN2B*/*CDKN2B-AS1* SNP variants and IOP.

Another locus associated with multiple optic disc endophenotypes and POAG is basic helix-loop-helix (bHLH) transcription factor atonal homolog 7 (*ATOH7*). Multiple GWAS analyses have shown an association of *ATOH7* SNPs with CA, DA, and vCDR quantitative structural traits in various cohorts [52, 55–60, 77–82]. The association between *ATOH7* and vCDR, however, is controversial as this observation may be related to the association of *ATOH7* with CA [54, 55, 59, 77, 80]. However, *ATOH7* SNPs have been associated with glaucoma in a number of studies [54, 55, 60, 65, 77, 79, 80, 82].

ATOH7 is a protein-coding gene located on chromosome 10 and encodes for a transcription factor that is essential for the genesis of RGCs [83–88]. RGCs are the earliest-born retinal cell type [90–92]. *ATOH7* mutations have been associated with optic nerve hypoplasia, while ectopic expression of *ATOH7* increases the number of differentiated RGCs in in vitro models [89–91]. *ATOH7* deletions have been reported to cause glaucoma, and *ATOH7* knockout mice lack RGCs and optic nerves [92].

GWASs have also identified SNPs in the region of the *SIX6* gene that are associated with glaucoma structural endophenotypes and POAG [58]. SNP rs10483727 (T-risk allele) and other *SIX6* SNPs are associated with increased vCDR, CA, and POAG [52, 56, 57, 59, 60, 67, 77, 79]. Interestingly, *SIX6* SNPs have specifically been associated with thinner RNFL thickness in both European Caucasian and Singaporean populations [93–95].

SIX6 codes for a homeobox protein found on chromosome 14 and is important for eye development, acting as a transcriptional repressor of the CDK inhibitors [77]. Evidence exists that *SIX6* regulates early retinal progenitor cell proliferation during eye development, and "risk" alleles subsequently cause retinal hypoplasia, with affected eyes exhibiting at least a 20 % decrease in RGCs by P35 (an essential activator of CDK5) [94, 96–101]. In addition to this pathway, it has been shown that knockdown of *SIX6* causes both a "small eye" phenotype and increases the expression of CDKN2b, linking the two loci as well as the TGF-β pathway and eye development [94]. Additionally, homozygous deletions of a genomic region containing *SIX6* are associated with variable degrees of retinal hypoplasia, the absence of the optic chiasm and optic nerve, and bilateral anophthalmia [78, 96, 102, 103].

Similar to *CDKN2B*/*CDKN2B-AS1* SNP variants, *SIX6* variants have been associated with established risk factors for POAG. Studies have suggested a link between SIX6 and IOP [104] and also myopia [105]. As with *CDKN2B*/*CDKN2B-AS1*, links between *SIX6* variants and physiological risk factors for glaucoma are encouraging in terms of uncovering additional pathways leading to POAG.

TGFBR3 and *CDC7* also code for proteins involved in the TGF-β signaling pathway. *CDC7* is located on chromosome 1 and codes for cell division cycle 7-related protein kinase, which is critical for G1/S transition. *CDC7* overexpression is reported to cause cell cycle arrest in S phase, and increased activity may aid recovery and repair of stalled replication forks, enhancing survival of tumor cells [106]. *CDC7* protein kinase can be both induced and activated by TGF-β during cell proliferation and differentiation, and lower levels lead to cell cycle arrest

and death in cancer cells [107, 108]. Transforming growth factor beta receptor III (*TGFBR3*) is the most abundant TGF-β co-receptor, and it may enhance the binding of TGF-β ligands to TGF-β type II receptors [109]. Expression of both *TGFBR3* and *CDC7* mRNA was observed in tissues relevant to POAG, such as the trabecular meshwork, optic disc, and optic nerve [72].

TGFBR3-CDC7 SNPs have been associated with visual field (VF) progression in Singaporean POAG patients [110]. VF tests assess the potential presence of blind spots (scotomas) in the eye, and visual function is frequently assessed using static automated perimetry. Optic nerve damage caused by POAG causes a very specific pattern of VF deficits. These functional deficits correspond to the anatomic distribution of the RNFL. If allowed to progress, glaucomatous field loss will result in functionally significant visual loss and affect quality of life. The detection of progression using this test is confounded by inter-test and intra-test variability, and the identification of true VF progressors can be difficult to detect [111]. Furthermore, obtaining both genetic and long-term VF progression data can be challenging and is not typically available for all cases studied in a GWAS. The association of *TGFBR3-CDC7* SNPs with VF progression is an important step in identifying new pathways that could lead to the development of biomarkers for early diagnosis and better screening for patients with higher rates of disease progression.

Although individual SNPs associated with optic nerve head endophenotypes may have minimal effects on glaucoma risk, these SNPs when aggregated in analysis with SNPs of other quantitative endophenotypes have stronger associations with glaucoma. Several studies have developed multi-locus "genetic risk scores" based on top SNPs. SNPs within the gene regions of *ATOH7*, *CDKN2B*, and *SIX1*/*SIX6* were more strongly associated with glaucoma when aggregated into a risk score [112, 113]. In another study, a risk score was developed using 7 IOP- and 18 vCDR-associated SNPs from a multiethnic Asian population in Singapore [114]. Subjects with higher genetic risk scores carried significantly increased risk of glaucoma and POAG, and this association was consistent across the three Asian ethnic groups [123]. The increased association with POAG for the combined SNPs may be due to increased statistical power compared to individual analysis [115, 116] but could also result from the polygenic nature of glaucoma where multiple genes affect multiple endophenotypes [113].

20.5 CCT

Central corneal thickness (CCT) is a highly heritable quantitative that can impact POAG pathogenesis, especially in patients with elevated IOP [117]. The heritability of CCT is very high with an estimated heritability of up to 95 % [118]. Thinner CCT may increase susceptibility to optic nerve damage; however, this association may be due, in part, to the effect of CCT on IOP measurement [117, 119].

GWASs have identified nearly 30 loci associated with CCT [120–122]. In GWASs conducted in Singapore, several collagen-related genes were associated with CCT in a Chinese, Malay, and Indian population including *ZNF469* (16q24), *COL5A1*/*RXRA* (9q34.2-q34.3), and *COL8A2* (1p34.2) [123, 124]. These genes have important roles in extracellular matrix metabolism, and rare mutations disrupting protein function are known to cause Ehlers-Danlos syndrome (*COL5A1*) [125], brittle cornea syndrome (*ZNF469*) [126–128], and Fuchs' corneal dystrophy (*COL8A2*) [129, 130]. *FNDC3B*, which was found to be associated with IOP and POAG in an Asian population [46], has also been associated with CCT. This gene appears to have a role in the regulation of cell motility and also has a role in TGF-β pathway activation in cancer [131]. In a Chinese POAG cohort, raftlin lipid raft linker 1 (*RFTN1*) was associated with CCT [82]. *RFTN1* SNPs have also been associated with Alzheimer's disease [132] and with vCDR and DA in a European Caucasian GWAS [55].

20.6 POAG

GWASs have identified important POAG susceptibility genes including *CAV1*/*CAV2*, *CDKN2B*/*CDKN2B-AS1*, *SIX6*, *TMCO1*, *ABCA1*, *GMDS*, *AFAP1*, *FNDC3B*, *PMM2*, *TGFBR3*, *GAS7*, *TXNRD2*, *ATXN2*, and *FOXC1* [43, 45–47, 50, 58, 72, 133, 134]. Asian cohorts have had a fundamental role in many of these studies.

POAG is generally less common in Asia than in the Western hemisphere. In Asians, prevalence of POAG varies, ranging from 1 to 4 % [135]. A recent study showed a POAG prevalence of 0.86 % in a Southwestern Chinese population aged ≥50 years [136]. Another study reported the prevalence of POAG in mainland China at 0.7 % with a higher prevalence in urban areas [137], while in India, the prevalence exceeds 1.7 % [138]. The higher prevalence of glaucoma in cities may be explained in part by the higher prevalence of myopia in urban areas [139]. Moderate to high levels of myopia conferred a two- to threefold increase in the risk of POAG among those with Chinese ancestry [140]. Most Japanese POAG patients have the NTG subtype with up to 92 % of POAG in Japanese characterized by normal IOP (≤21 mmHg) [141]. Despite the lower POAG prevalence overall, Asia currently has the largest absolute number of glaucoma cases worldwide due to sheer population size and will have the largest number of persons living with POAG and PACG worldwide by 2020 [1].

A GWAS using a Chinese discovery cohort with replication in Singaporean Chinese recently identified significant association with POAG and SNPs in the *ABCA1* and *PMM2* regions [47]. *ABCA1* was also identified in a European Caucasian GWAS [45]. While *PMM2* has not demonstrated genome-wide association in any other GWAS yet, interesting association with SNPs in this region was observed in another recent study of European Caucasians [50].

One of the first GWAS for POAG identified variant rs4236601[A] associated with disease risk in east Asians and Europeans. This SNP is in the intergenic region between *CAV1* and *CAV2*, which are both expressed in most human cell types, including scleral spur cells [142], the trabecular meshwork [143], and RGCs [133, 144]. *CAV1* and *CAV2* are involved in formation of caveolae, which are lipid- and cholesterol-rich invaginations of the plasma membrane that participate in transcytosis [145]. Interestingly, in an experimental setting, *CAV1* was found to be upregulated in the trabecular meshwork after 1 h of increased IOP [146]. Additionally, *CAV1* is an important regulator of nitric oxide synthase (eNOS) [147, 148] and TGF-β signaling; and both of these signaling pathways are implicated in the pathogenesis of POAG [149, 150].

A multiethnic study of POAG using a Singaporean Chinese cohort as the discovery sample identified *TGFBR3* as a susceptibility locus. The lead SNP in this region has also been associated with rate of disease progression in the Singaporean Chinese cases [72, 110]. More recently a POAG GWAS identified *TXNRD2* and *FOXC1* as susceptibility loci for POAG in Caucasians of European ancestry with replication in Singaporean Chinese [50].

As noted previously, NTG is very prevalent in Asian populations, especially Japanese. The first GWAS completed for NTG was in the Japanese population identifying SRBD1 and ELOVL5 as NTG susceptibility loci [151]. These genes are involved in apoptosis or cell growth inhibition; and the regulation of SRBD1 and ELOVL5 cascades could play a key physiologic role in the pathogenesis of NTG, such as affecting the survival of RGCs [141, 151].

20.7 Angle-Closure Glaucoma

PACG remains a major cause of irreversible blindness throughout Asia [152–155] with largely unresolved causal mechanisms. Nearly 80 % of the estimated 15 million people afflicted with PACG reside in Asian countries [156], and it is estimated that PACG blinds proportionally more people than POAG globally [157]. Population-based prevalence studies found a higher incidence of PACG in Asians than in those of

European ancestry [158] and blinds up to ten times more people than POAG in Asian populations [159].

Anterior chamber depth (ACD) is a major risk factor for PACG, with small anterior segment dimensions or shallow ACD being the cardinal feature associated with increased susceptibility to PACG [160–163]. ACD is easily measured by ocular imaging modalities and is a normally distributed within the general population [164]. As with other quantitative endophenotypes, ACD displays high heritability with a coefficient as high as 0.90 [165, 166].

GWASs have been useful in identifying sequence variants that confer susceptibility to PACG. One GWAS identified SNP rs1401999 at *ABCC5* associated with ACD and PACG risk in Indians, Malays, Chinese, and other Asian cohorts [164], but did not find significance for previously identified PACG-associated and ACD SNPs [167, 168]. *ABCC5* has been previously associated with eye development in fish [169] and is known to be expressed in the human eye [170, 171]. However, its role in the context of PACG is not yet known. Another GWAS studying Malay, Chinese, Vietnamese, Indian, and Singaporean cohorts identified three loci for PACG, including SNPs rs11024102 in *PLEKHA7*, rs3753841 in *COL11A1*, *and* rs1015213 located on chromosome 8q [167]. *COL11A1* encodes for one of the two type XI collagen α chains and is expressed in human ocular trabecular meshwork cells [172]. The gene has previously been associated with multiple syndromes causing ocular, orofacial, auditory, and skeletal abnormalities [173]. *PLEKHA7* encodes pleckstrin homology domain-containing protein 7. This protein is essential for the integrity of adherens junctions, which control epithelial and endothelial paracellular permeability [174–176] and provide a barrier for fluid leakage in the choroid, ciliary body, iris, and aqueous humor outflow system [177]. This gene could influence PACG development by causing aberrant fluid dynamics, abnormal choroidal expansion, or reduction of iris volume [178–180]. Very recently a large multiethnic study identified five new PACG loci, and two of these new genes are also involved in cell adhesion (*FERMT2*, *EPDR1*) suggesting that this is an important mechanism underlying PACG pathogenesis [181]. These and other insights into PACG's genetic architecture provided by GWASs could aid in the development of a genetic profile for the identification, risk stratification, and treatment of patients with PACG.

20.8 Exfoliation Syndrome Glaucoma

Exfoliation syndrome (XFS) is an age-related disorder of the extracellular matrix that is characterized by the production, deposition, and progressive accumulation of a cross-linked, amyloid-like fibrillar material on the pupillary border and anterior lens surface. Exfoliation glaucoma is the most serious known complication of XFS [182]. The deposition of XFS pigment in the trabecular meshwork can lead to decreased outflow of aqueous fluid from the eye, resulting in elevated IOP and glaucoma. XFS is the most common identifiable cause of open-angle glaucoma worldwide and comprises the majority of glaucoma in some countries [183].

In 2007, the first GWAS on XFS identified lysyl oxidase-like 1 (*LOXL1*) on chromosome 15q22 as a major susceptibility locus for XFS, finding three *LOXL1* SNPs strongly associated with exfoliation glaucoma in individuals of European, African, and Asian ancestry [184]. *LOXL1* codes for an enzyme that is essential for the formation, maintenance, and remodeling of elastic fibers and prevents age-related loss of tissue elasticity. The LOXL1 enzyme is a major component of exfoliation deposits and plays a role in its subsequent accumulation in the eye [185]. The association of *LOXL1* with XFS has been confirmed in populations from throughout the world including Asian populations of Chinese, Indian, and Japanese [186–205]. Defining the precise role of *LOXL1* in XFS will require additional studies, including deep re-sequencing efforts to further characterize specific risk alleles [206].

A recent XFS GWAS using a Japanese cohort as the discovery sample identified *CACNA1A* as a susceptibility locus for XFS [186]. *CACNA1A* encodes for a subunit of the type P/Q voltage-dependent calcium channel and is expressed in many ocular tissues [186]. Calcium channels transport calcium ions across cell membranes and help to generate and transmit electrical signals. Altered calcium channel function could lead to the formation of XFS aggregates by altering calcium concentrations [207] and interfering with fibrillin's ability to form stable aggregates [208].

20.9 Conclusion

GWASs have revealed many novel loci associated with POAG, PACG, NTG, XFS, and disease-related quantitative traits. Despite this success, the total number of glaucoma-related loci remains far fewer than other ocular diseases with complex inheritance such as age-related macular degeneration [209]. Future studies are expected to continue to uncover new glaucoma loci, and those studies that include multiethnic populations are likely to produce the most interesting results. In addition to large multiethnic studies, ascertaining cases or controls at the extreme ends of the phenotypic spectrum may be a useful approach; more severely affected individuals could have a larger number of "risk" variant SNPs or SNPs with larger effect sizes [210].

In glaucoma, where damage is irreversible and cases often go undiagnosed until severe visual loss occurs, having the ability to accurately predict and detect disease prior to symptomatic vision loss is critical [211]. The identification of genes that predispose an individual to glaucoma will allow us to further understand disease mechanisms and identify individuals at risk at early stages of disease when therapy is most effective. Currently, translation of genetic findings to clinical practice is limited, but with the discovery of a comprehensive set of glaucoma predisposing genes, clinically useful gene-based tests may be possible. Moreover, the identification of genes contributing to glaucoma could point to important biological pathways involved in the disease pathogenesis that may be targets for novel, and ideally neuroprotective, therapies.

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Genetic Complexity of Primary Angle-Closure Glaucoma in Asians

21

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Abstract

Glaucoma is a group of optic neuropathies, characterized by progressive loss of axons in the optic nerve and degeneration of retinal ganglion cells accompanied by visual field loss. It is the second foremost irreversible cause of blindness after cataract worldwide. Even though primary open-angle glaucoma (POAG) is a more prevalent form of glaucoma, primary angle-closure glaucoma (PACG) accounts for substantial cause of visual morbidity worldwide. PACG is one of the subtypes of glaucoma with multifactorial aetiology characterized by the opposition of the iris to the trabecular meshwork, thus hindering aqueous outflow from the angle of an eye. It is more common among the people of Asian descent than Europeans. It is a heterogeneous disorder with complex genetic basis affecting many individuals within a family. Since, it is often asymptomatic in early stages and its late onset nature, diagnosis is frequently delayed. Therefore, understanding the genetic aspects and identification of risk factors will shed light to unravel the complexity of this complex disorder. Several genetic approaches and molecular biology techniques can be used to comprehend and identify susceptibility loci for angle-closure glaucoma. This chapter gives insights into the current status concerning epidemiology, risk factors, disease classification, its mechanism and genetics of angle-closure glaucoma.

Keywords

PACG • Genetics • GWAS • SNP

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Abbreviations

PACG	Primary angle-closure glaucoma
PAC	Primary angle closure
PACD	Primary angle-closure disease
SNPs	Single nucleotide polymorphisms
GWAS	Genome-wide association studies
MMPs	Matrix metalloproteases
HGF	Hepatocyte growth factor
MFRP	Membrane-type frizzled-related protein gene
PRSS56	Serine protease 56
MTHFR	5,10-methylenetetrahydrofolate reductase
CALCRL	Calcitonin receptor-like receptor
ECM	Extracellular matrix
HSP70	Heat shock protein 70 kilodalton
eNOS	Endothelial nitric oxide synthase

21.1 Introduction

Glaucoma is a complex neurodegenerative disorder characterized by progressive retinal ganglion cell damage, loss of optic nerve axons and distinctive optic neuropathy. It leads to optic disc cupping and concomitant loss of visual field and eventually blindness, if untreated. According to the recent World Health Organization (WHO) data, the major causes of global blindness are cataract (51%), glaucoma (8%), age-related macular degeneration (AMD, 5%), childhood blindness and corneal opacities (4%) [1]. Glaucoma represents the second most leading cause of blindness in the world following cataract. It can remain asymptomatic and undiagnosed for a longer time period in its early stages, resulting in a more severe form at later advanced stages with more number of affected people. In general, glaucoma is broadly classified into three major groups: (i) primary open-angle glaucoma (POAG), (ii) primary angle-closure glaucoma (PACG) and (iii) primary congenital glaucoma. Based on the anatomy of anterior chamber angle, primary glaucoma can be classified into POAG and PACG. PACG results from an appositional contact between peripheral iris and trabecular meshwork causing partial or complete anterior angle closure and thus hindering aqueous outflow. This leads to an increase in intraocular pressure (IOP) and damage to the optic nerve. It is a genetically complex disorder and more common among people of Asian descent. Eighty-six percent of people with PACG are in Asia, with approximately 48.0% in China, 23.9% in India and 14.1% in Southeast Asia [2]. These numbers highlight the importance of understanding the disease, its classification, approaches to study the risk factors and genetics of angle-closure glaucoma.

21.2 Epidemiology of Primary Angle-Closure Glaucoma and Asian Perspective

Several epidemiological studies in glaucoma provide us with insights into the amount of disease and its risk factors. Asia is the largest continent in the world with around 4.3 billion population that accounts for 60% of the world total. According to the recently published meta-analysis by Tham et al., the global prevalence of glaucoma for population aged 40–80 years is 3.54%, and the prevalence of PACG is highest in Asians (1.09%) [3]. The blindness caused by glaucoma is expected to reach alarming proportions. In 2013, the number of people with glaucoma was estimated to be 64.3 million, increasing to 76 million in 2020 and 111.8 million in 2040, and Asia will have the largest number of persons affected by PACG worldwide [3]. Growth in glaucoma prevalence is mainly the result of expected changes in the number of older persons. Although the number of elderly persons is likely to increase in Europe and North America, it is expected to increase more dramatically in Asia because of increased life expectancy in this region.

PACG, despite being less prevalent than POAG, is responsible for most of the blindness in India [4]. In most of the population-based studies in India, PACG was observed to cause one to four times the proportion of blindness as

POAG [4, 5]. It is also responsible for most of the uncorrectable bilateral glaucoma blindness in Mongolia and Singapore [6, 7]. It has been estimated that by 2020 there will be 5.3 million people blind from PACG [2]. Based on the estimates from China, the proportion of those blind with PACG is 25%, double that of POAG blindness, which is 10% [8]. All these studies stress the importance of understanding the need for early disease detection and intervention.

21.3 PACG Disease Definition and Classification

Primary angle closure has been defined as appositional or synechial angle closure of the anterior chamber angle caused by pupillary or other features.

21.3.1 Classification

The best method of classifying the various types of angle-closure glaucoma has long been debated. Foster and associates have published a classification of angle closure that has become widely used in prevalent studies and epidemiological research – International Society of Geography and Epidemiology of Ophthalmology (ISGEO) classification [9]. It specifies three stages, each of which are described in greater detail below:

Primary angle-closure suspect (PACS)
Primary angle closure (PAC)
Primary angle-closure glaucoma (PACG)

21.3.1.1 Primary Angle-Closure Suspect

The individual with an anatomically narrow angle can be classified as PACS when gonioscopy reveals a shallow peripheral angle recess with the iris apposed, but not stuck to, the trabecular meshwork. PACS has iridotrabecular contact (ITC) in two or more quadrants, no peripheral anterior synechiae (PAS) and normal intraocular pressure (IOP), optic nerve and visual field.

21.3.1.2 Primary Angle Closure

In PAC, the individual has ITC in three or more quadrants, along with elevated IOP and/or primary PAS, but a normal optic nerve and visual field.

21.3.1.3 Primary Angle-Closure Glaucoma

In PACG, the individual has the features of PAC, with glaucomatous optic neuropathy and a corresponding visual field defect compatible with glaucoma.

This classification is based on the presumed natural history of angle-closure glaucoma and constitutes a spectrum ranging from anatomical narrowing (PACS) to PAC where there is evidence of trabecular damage to PACG, where there is additional functional damage in the form of optic neuropathy.

21.4 Aetiology and Mechanism of Primary Angle-Closure Glaucoma

The major mechanism acting in primary angle closure is pupil block. Although the reason for the same is debated, it is the result of a narrow channel between the iris and lens as a result of an anteriorly lens position, high lens vault or a thick lens; it is widely regarded to be the result of simultaneous activation of dilator and sphincter pupillae muscles. Other factors can produce angle closure without pupillary block including anteriorly positioned ciliary body, thick iris and iris which does not lose volume with dilation.

Quigley et al. also proposed choroidal expansion as a contributing risk factor for PACG and acute primary angle closure (APAC) [10]. The degree to which choroidal expansion occurs depends on many variables including choroidal elasticity and vascular permeability.

Primary angle closure not only results from predisposing anterior segment anatomy but also unfavourable physiological behaviour [10]. Quigley proposed that the normal iris acts as a ‘sponge’, and it loses volume on dilatation

by losing fluid to the aqueous. Further evidence to support the concept of abnormal physiological behaviour comes from an ocular coherence tomography (OCT) study where iris volume was found to increase significantly following pharmacological dilatation of the pupil, in the contralateral eyes of patients with previous APAC, while it decreased significantly in those of age- and gender-matched controls [11].

21.5 Risk Factors

21.5.1 Anatomical Risk Factors (Biometry)

Cross-sectional and clinical studies consistently find that small eyes with shallow anterior chambers, short axial lengths, hyperopic refractive errors, small corneal diameters, shallow limbal chamber depths and thick, relatively anteriorly positioned lenses are at risk for angle closure [12].

The width of angle is a commonly used parameter to grade the risk of angle closure. Gonioscopic examination remains the most important method for grading the angle width and describing other signs of angle closure. Several gonioscopic grading methods have been devised, which include Scheie, Shaffer and Spaeth systems. All these systems are based on estimation of the width of the angle in degrees or visibility of angle landmarks. Gonioscopic grading is a subjective measurement that usually depends on examiner's experience and skill.

Anterior segment imaging (or) biometric technologies, such as ultrasound biomicroscopy (UBM), A-scan ultrasound biometry, IOL Master, scanning peripheral anterior chamber depth analyser (SPAC) and anterior segment optical coherence tomography (AS-OCT), all provide objective means of quantifying the angle. SPAC, AS-OCT and IOL Master are fast, non-contact methods and therefore may be more suitable for large-scale screening. However, these methods are not able to consistently identify the pigmented trabecular meshwork and cannot ultimately tell if there is iridotrabecular contact – the hallmark of angle closure.

A simple dichotomous classification of width of angle has been used in epidemiological studies, in which angles have been termed 'occludable' or 'not occludable' based on a number of quadrants (normally two or three quadrants) with trabecular meshwork not visible [9].

21.5.2 Age, Gender and Ethnicity

Increasing age is a risk factor for primary angle closure disease (PACD). The decrease in anterior chamber depth in older individual is likely primarily due to thickening and anterior movement of the lens [13]. Female gender is a major predisposing factor for PACD. The prevalence of all categories of angle closure is 2–5 times higher in women than in men. This may due to shallow anterior chamber in women [14]. Ethnic differences have been well recognized in PACD. Both prevalence and incidence of PACD are high in East Asian population than European [15]. This ethnic difference may be attributed to differences in anterior chamber and angle anatomy. The majority of evidence suggests there is an inverse association between ACD and rate of angle closure in various ethnic groups.

21.5.3 Family History

A positive family history has long been recognized as predisposing factor to angle closure [16]. The similarities of ocular biometry in first-degree relatives of angle closure indicate that angle closure-related anatomical characteristics are heritable. Sihota et al. examined first-degree relatives of Indian PACG patients and reported a prevalence of 36.9% of PACG among siblings [17]. Kavitha et al. compared the prevalence of angle closure among the siblings of patients with open angles (OA), primary angle-closure suspect (PACS) and either primary angle closure (PAC) or PACG. Angle closure was more prevalent in both PACS siblings (35%) and PAC/PACG siblings (36.7%) compared with open-angle

siblings (3.7%) [18]. Also, the risk was more in women and older sibling. In addition, the study showed that the siblings of patients with angle closure are at 8.8 times greater risk of angle closure.

21.5.4 Genetics

Genetic factors have been extensively studied for their role in the pathogenesis of PACD. A genetic aetiology for PACG is supported by several epidemiological and clinical studies which showed familial aggregation and higher risk of disease in family members [17, 18] as well as heritability of risk factors such as anterior chamber depth [19]. All these factors strongly suggest that genetic factors are involved in the pathogenesis of PACG. The successful identification of novel PACG-associated genes has provided insights into the genetic mechanisms underlying the disease susceptibility. The detailed genetic studies on PACG are described under Sect. 21.9.

21.6 Clinical Features

21.6.1 Symptoms

Patient with acute angle-closure attacks may go from a state of white and comfortable eye to a red, painful eye. It is often associated with headache, nausea, vomiting and haloes. Upon inquiry history of one or several episodes of altered vision, discomfort and haloes can be obtained. The diagnosis of acute angle closure is usually made from clinical history and a careful gonioscopic examination. Indentation gonioscopy with four mirror lens is absolutely essential to determine if elevated IOP is from angle closure or other features.

21.6.2 Signs

Patient with acute angle closure typically presents with ciliary congestion, corneal oedema, elevated intraocular pressure, shallow anterior chamber or mid dilated fixed or sluggish reacting pupil. Marked raise in intraocular pressure can result in glaukomflecken, which are localized lens opacities due to pressure-induced necrosis. Following an acute congestive episode, one can find atrophic changes in iris, glaucomatous optic neuropathy. These eyes may have significantly lower corneal endothelial cell count compared to unaffected fellow eyes [20]. Examination and treatment of fellow eye (i.e. with prophylactic laser iridotomy) are essential in preserving vision [21].

21.7 Understanding the Basic Human Genetics

It is well established that human characteristics are inherited from parents to offspring. The genetic information of an individual is contained in 23 pairs of chromosomes. Every human cell contains the 23 pair of chromosomes. One pair is called sex chromosomes (i.e. in males, XY and in females, XX), and other 22 pairs of homologous chromosomes are called autosomes. Two chromosomes in the same pair are called homologous chromosomes. For every pair of chromosomes which contains DNA molecule, one is inherited from the mother, and other is inherited from the father of an individual with equal probability. A gene is the most fundamental unit of heredity found on chromosomes that controls the transmission and expression of one or more traits. The chemical structure of a gene is deoxyribonucleic acid (DNA). An organism's basic complement of DNA is called its genome. DNA is a double-helical structure made up of subunits called as nucleotides. Each nucleotide consists of a phosphate group, a deoxyribose sugar and one of four different nitrogenous bases: adenine (A), guanine (G), cytosine (C) or thymine (T). The DNA backbone is a polymer with an alternating sugar-phosphate sequence. A codon is a triplet of nucleotides which codes for a specific amino acid. This linear sequence of contiguous triplets gives an amino acid sequence of a protein. A DNA acts as a template and transcribed into an mRNA (messenger RNA), which, in turn, translated into the specific protein by

the process of translation. Genes actually do not form a continuous sequence but consist of several coding segments called exons that are separated by non-coding segments called introns. These introns are removed from the pre-mRNA molecule through RNA splicing, and only the exonic (the coding sequences) regions of a gene form the final messenger RNA (mRNA) and finally get translated into protein. In addition, there are other factors (transcription factors, enhancers, repressors, etc.) involved in regulating the expression of genes.

For perfect functioning of the human organism, stability and constancy of the genetic information are essential. But transfer of genetic information is very susceptible to error or alteration which can have awful consequences. The change in the hereditary substance may take place at chromosome level or in the DNA itself. Such alterations are called as mutations. Besides, gene mutations, other variations observed at single nucleotide level (or single base pair changes) are known as single nucleotide polymorphisms (SNPs) which are one of the most common types of genetic variation. SNPs are bi-allelic (consists of two alleles) sequence variants that are ubiquitously distributed across the genome whether they are coding, non-coding and untranslated regions (UTRs). SNPs are conserved during evolution and account for over 90% of an individual's genetic variations, occurring at a frequency of greater than 1% in the population. Some of the SNPs are located in the coding regions causing substitution of one amino acid to other and thus altering or hampering protein function. Moreover other SNPs located in the non-coding and regulatory regions can perhaps modify gene function and disrupt gene expression [22].

Genetic diseases are divided broadly into four types: single gene defects, complex (multifactorial or polygenic) diseases, chromosomal abnormalities and mitochondrial diseases. Chromosomal diseases are caused by alterations in chromosome structure or number (e.g. Down syndrome, Turner syndrome). Mitochondrial diseases may be caused by mutations in the mitochondrial DNA (mtDNA) or in nuclear genes coding for components of mitochondrial affecting mitochondrial function (Leber's hereditary optic neuropathy, LHON). Single gene defects are also known as Mendelian inheritance disorders in which alterations in a single gene are responsible for a defect (e.g. cystic fibrosis, sickle cell disease). In contrast, complex diseases result from a combination of genetic variations as well as environmental factors which together modulate the susceptibility, severity and clinical manifestation of the disease.

Finding the genetic basis of a complex disease is considerably challenging and is one of the most active areas of current research. Consequently, completion of Human Genome Project enabled researchers to look for the complex diseases to disentangle the genetic pathogenesis using various genetic approaches. Therefore, understanding the basic human genetics and different genetic approaches allows us to understand the complex involvement of genes responsible for the disease and to further unravel the mysterious areas in this field.

21.8 Genetics and Molecular Biology Approaches to Map PACG

The precise genetics of angle-closure glaucoma is not clearly established, but it is likely to be a heterogeneous disorder involving interactions of many genes and environmental factors. Besides genetic and environmental factors, various other factors like oxidative stress, lifestyle modification, etc. also play a pivotal role in the pathogenesis. There are various genetic and molecular biology approaches which can be used to understand disease aetiology at a molecular level such as candidate gene approach, genome-wide linkage analysis, etc. for deciphering the genetic complexity of PACG.

21.8.1 Approaches for Mapping PACG

21.8.1.1 Gene Mutation Analysis

This approach is based on established studies (or prior knowledge) of a putative gene having known role in disease mechanism and aetiology.

Screening of putative gene for mutations is performed by bidirectional sequencing. It is one of the most widely used approaches employed in the dissection of the genetic basis of a disease. The pathophysiology of PACG is less understood and no specific gene has been identified for PACG, therefore, mutation analysis is less efficient in establishing the genetic basis of the disease. Rather, genome-wide studies prove to be more efficient and useful to explore more and identify new susceptibility loci for PACG.

21.8.1.2 PCR-RFLP

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) is a cost-effective method for SNP genotyping in genetic association studies. In this, target region containing variation is PCR amplified using specific primers, and then amplified fragment is treated with specific enzymes known as restriction enzymes. Based on the restriction enzyme recognition site, it results in the formation of fragments of different sizes. Simultaneously, genotype of an individual can be known by electrophoretic movement of the fragments on agarose gel. But this technique is not suitable for high-throughput analysis because it does not allow simultaneous analysis of a large number of SNPs at a single time since it requires specific primer pair and restriction enzyme for each SNP.

21.8.1.3 TaqMan Allelic Discrimination Assay

High-throughput methods are extremely helpful for genotyping SNPs to perform large-scale association studies. TaqMan assay is a PCR-based assay for genotyping single nucleotide polymorphisms (SNPs). The flanking region near SNP is amplified in the presence of two allele-specific fluorescent probes. The probes do not fluoresce in solution because of a quencher at the 3′ end of a DNA strand. The presence of two probes allows the detection of both alleles (for genotyping) at a given time. This assay can be multiplexed, i.e. detection of up to seven SNPs in one reaction can be possible. TaqMan assay is a robust, cost-effective and high-throughput technique for performing association studies like population-based association analysis for various complex disorders like glaucoma, AMD, diabetic retinopathy, etc.

21.8.1.4 Gene Association Studies

Genetic association studies offer a powerful approach for identifying genes or regions in the genome that confers disease susceptibility, especially for complex disorders [23]. The main purpose of the association studies includes determining the association between a genetic variant (SNP) with disease and also whether an individual having high-risk variant for a specific trait will develop disease with increased risk [24]. Usually, these studies used a case-control design; however, family-based designs are also used based on the need of the study. Because of widespread occurrence of the SNPs in high frequency, these are believed to be the potential candidates for disease susceptibility and have been used as markers in genetic association studies.

21.8.1.5 Genome-Wide Association Studies (GWAS)

Genome-wide association studies (GWAS) is a cost-efficient approach to understand the genetic basis by identifying the associated SNPs or genetic variants linked to complex disease phenotypes. Human genome sequence consists of numerous variations and SNPs are the most common one. GWAS involves screening several hundreds of thousands SNPs across the genome responsible for disease susceptibility in a very large number of sample set (e.g. cases and controls). It has a potential to divulge novel genes which are previously not identified by other approaches or earlier not associated with disease aetiology [25], but requires a large study population. Once such markers are identified through GWAS, the results need to be replicated in different populations in independent sets of samples. Thus, GWAS provides us the way to understand the disease pathogenesis and increases our knowledge regarding complex disorders [26].

Basic methodology for evaluating genetic association between potential loci (or particular SNP) and disease is as follows (see Fig. 21.1):

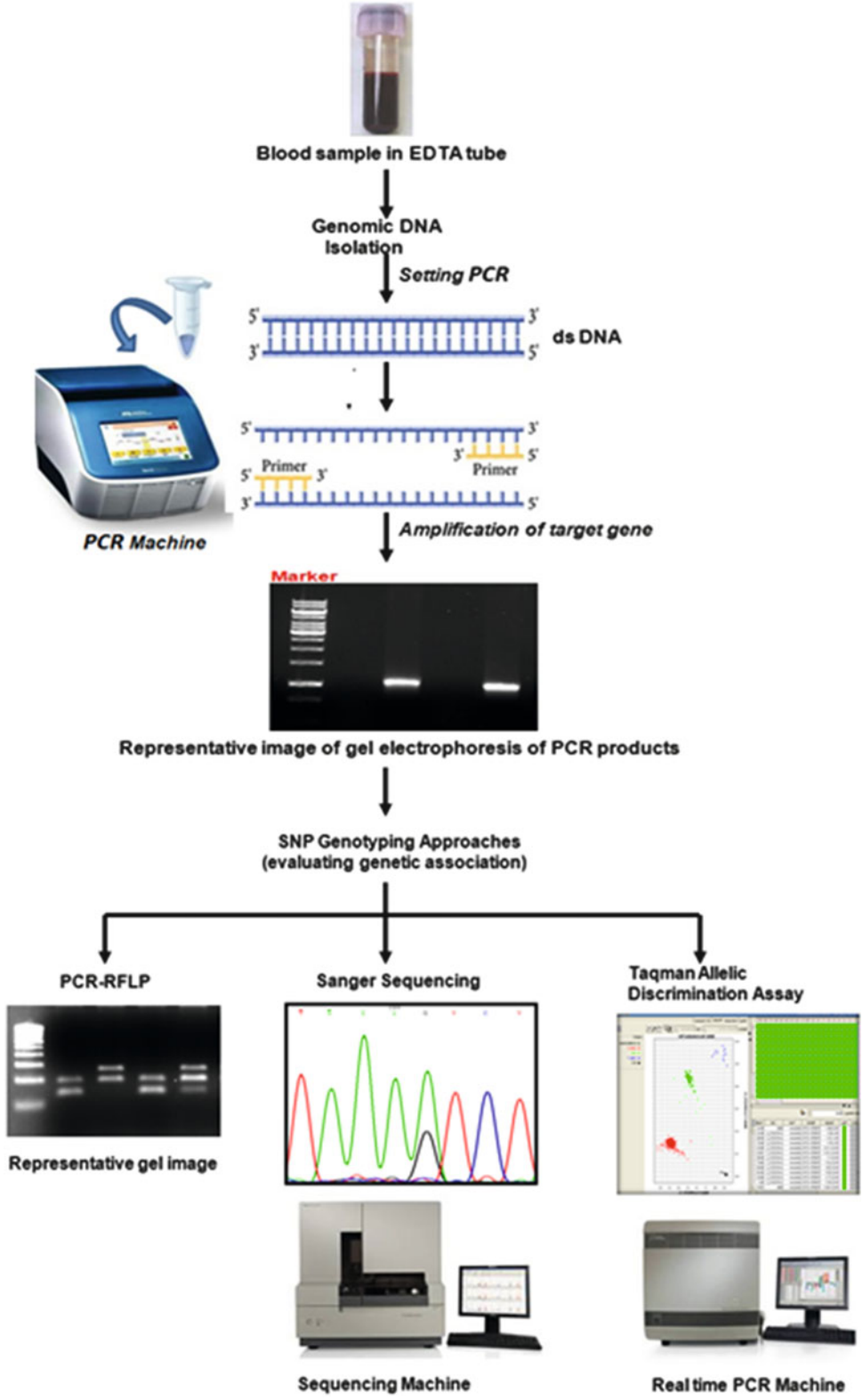

Fig. 21.1 Basic methodology for SNP genotyping

1. Collection of blood sample from study subjects.
2. Genomic DNA isolation from the subject's whole blood.
3. Set-up of polymerase chain reaction (PCR) using a set of primers specific for a gene or particular SNP to check for genetic association.
4. RFLP analysis using restriction enzymes specific to check the genotype of patient for the SNP at a locus.
5. Simultaneously, confirmation can be done by Sanger sequencing.

21.9 Genetic Studies on PACG

PACG shows many signs of being a heterogeneous disorder with both genetic and environmental aetiological factors though its genetic basis is not well understood. Genetic factors represent a part of the risk associated with complex disease phenotypes. Certainly, the interplay between genetic and environmental factors in complex disorders is persistently challenging researchers [27]. Several studies have suggested a genetic basis for PACG [16, 28]. Even though 25 loci have been linked to POAG till date with three underlying genes [29], the whole genes underlying PACG are still unknown. This section of the chapter focuses on the various genetic studies, and their results till now are published with reference to angle-closure glaucoma primarily in Asian population.

21.9.1 Genetic Association Studies

In recent years, PACG has become an important target for association studies using SNPs as markers. Several SNPs in the candidate genes such as matrix metalloproteases (MMPs), hepatocyte growth factor (HGF), membrane-type frizzle-related protein (MFRP) gene, serine protease 56 (*PRSS56*), 5,10-methylenetetrahydrofolate reductase (MTHFR), calcitonin receptor-like receptor (CALCRL) and so on have been reported in different ethnicities with PACG which are explained successively in this section.

21.9.1.1 Matrix Metalloproteases (MMPs)

There are several anatomical characteristic features (such as shorter axial length, AL) associated with PACG eyes that act as predisposing factors along with the genes for the development of angle-closure glaucoma. Since the elongation of eye is closely related to extracellular matrix (ECM) remodelling of the connective tissue, understanding the ECM composition and remodelling process is essential. ECM is an intricate network of molecules secreted by the cells that provides structural and biochemical support to the surrounding cells. Abnormalities in the ECM and its remodelling have been associated with glaucoma and are likely to be a vital determinant for the short axial length in relatively small eyes. ECM is composed of meshwork of two major classes of biomolecules: proteoglycans (PGs) and fibrous proteins [30, 31]. These proteoglycans are made up of glycosaminoglycans (GAGs) which are carbohydrate polymers often attached to proteins to form proteoglycans (e.g. heparan sulphate, chondroitin sulphate and keratin sulphate) [32]. Fibrous proteins include collagens (type I, II, III, V, XI), elastins, fibronectins, etc. Degradation of ECM is required for tissue remodelling associated with various physiological processes such as morphogenesis, angiogenesis, tissue repair, etc.

Matrix metalloproteases (MMPs) are the major proteases involved in ECM degradation. MMPs are the zinc-dependent endopeptidases capable of degrading various kinds of ECM proteins, thereby playing a key role in tissue remodelling under normal as well as pathological conditions [33]. They are regulated by growth factors, cytokines, hormones and interaction with ECM molecules too. In addition, the activity of these MMPs is taken cared of by their endogenous inhibitors known as tissue inhibitor of metalloproteinases (TIMPs) [33]. MMPs can be divided into five main classes based on substrate

specificity and protein structure features: collagenases (MMP-1, MMP-8, MMP-13 and MMP-18), gelatinases (MMP-2, MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), membrane type (MMP-14, MMP-15, MMP-16, MMP-17) and others (MMP-7, MMP-12, etc.) [33]. MMP genes *MMP-1*, *MMP-3*, *MMP-7*, *MMP-8*, *MMP-10*, *MMP-12*, *MMP-13*, *MMP-20* and *MMP-27* are located on chromosome 11. MMP-9 is a 92 kilodalton type IV collagenase and the most studied gene particularly in relation to glaucoma which maps to chromosome 20 with 13 exons [34]. It cleaves denatured collagen and type IV collagen in the basement membrane [35]. Abnormal expression of MMPs has been already associated with glaucoma [36, 37]. Therefore, MMPs are the candidate genes to study for the shorter axial length in relevance to PACG. Polymorphisms in MMP genes might have some effect in function of *MMP* and thus affect ECM remodelling process.

First case-control study was performed by Wang et al. to study the association between various SNPs of *MMPs*, TIMPs and other glaucoma-associated genes and acute PACG in Taiwanese population [38]. But, out of all, only *MMP-9* SNP rs2664538 showed association with acute PACG which needs to be further studied to elucidate its exact role in disease pathogenesis. However, the same SNP rs2664538 in *MMP-9* did not show association with PACG (either acute or chronic) in Singaporean subjects [39]. Later, Cong et al. also performed association analysis between the two polymorphisms in *MMP-9* (rs2250889, C/G and rs17576, A/G) and PACG in southern China [40]. This study concludes that rs2250889 might be associated with PACG (P=0.004), and people with G/G genotype are probably more susceptible to PACG. However, rs17576 might not be related to PACG in southern Chinese cohort.

Shi et al. also screened some genes involved in tissue remodelling pathway such as *MFRP*, *HSP70, MTHFR* and *MMP-9* in relation to PACG. Studies on *MFRP* (see Sect. 21.9.1.4), *HSP70* (see Sect. 21.9.1.5) and *MTHFR* (see Sect. 21.9.1.7) will be explained in subsequent sections [41]. Here, in this study, association of the *MMP-9* SNPs rs17576 and rs3918249 with PAC subjects in Han Chinese population was investigated. This study did not find any association of these two SNPs with PAC that may be due to difference in distribution of SNPs among different ethnic groups and difference in sample size. Immediately after this study, Michael and co-workers also investigated the role of *MMP-1* (rs1799750), *MMP-7* (rs11568818) and *MMP-9* (rs3918242 and rs17576) SNPs with POAG and PACG in Pakistani population [42]. This study revealed significant association of MMP-1 rs1799750 with POAG (p=0.001) and MMP-9 rs17576 (p<0.001) with PACG. For POAG patients, significant association was observed for female subjects (p<0.001, but not for male subjects (p>0.47). Statistical analysis with gender stratification in PACG cases, a significant association was observed in male cases (p=0.009), but not for females (p=0.14). It shows that further study might help to check these SNPs as gender-specific risk factors in the development of PACG and POAG in Pakistani cohort. SNP rs17576 substitutes a positively charged arginine by an uncharged glutamine (at position 279) in a highly conserved gelatinase-specific fibronectin type II domain (FN2) [43, 44]. This domain is responsible for the collagen affinity of MMP-9 [45]. Precise mechanism on how this polymorphism affects the protein function and stability is unknown, but it is hypothesized that it might lead to partial loss of function in ECM remodelling that occurs during eye growth and development [38]. No significant association was observed for *MMP-7* (rs11568818) and *MMP-9* (rs3918242) polymorphisms in Pakistani population.

Recently, genetic association between the *MMP-9* SNPs (rs3918249, rs3918254, rs17576 and rs3787268) and PACG was evaluated in a Han Chinese population [46]. The SNP rs3918254 was reported as a more susceptible locus for PACG patients with CC and CT genotype, whereas the other SNPs rs3918249, rs17576 and rs3787268 showed no association with increased PACG risk in Chinese Han population. In short, further studies are necessary to

understand the mechanism which will shed light to further explore the role of these polymorphisms in the pathogenesis of PACG.

21.9.1.2 Hepatocyte Growth Factor (HGF) Gene

Hepatocyte growth factor (HGF) also known as scatter factor is a pleiotropic growth factor mainly expressed in mesenchymal cells, including fibroblasts, macrophages, etc. HGF plays a role in embryogenesis, tissue repair and angiogenesis. It regulates cell growth, cell motility, matrix invasion, migration of various epithelial cells, vascular endothelial cells and many other cell types and acts as a multifunctional cytokine on cells of mainly epithelial origin. Patients with severe hypertension show higher serum levels of HGF [47]. HGF appears to be an important modulator of cell growth and differentiation in the eye. Several studies demonstrated that it stimulates the growth and migration of corneal epithelial and endothelial cells, lens epithelial cells and trabecular cells [48–54]. HGF has been reported in variety of ocular fluids such as aqueous humour, vitreous humour and tears of different eye diseases [48, 55–59]. Veerappan et al. investigated the association of *HGF* with hyperopia and in regulating human ocular development (emmetropization process) [60]. As PACG and hyperopia share the same anatomical feature of short axial length, therefore, people started looking for HGF gene polymorphisms for PACG susceptibility [61].

First study was conducted by Hu and Ritch in which they measured the concentration of HGF in the aqueous humour from the glaucoma patients including PACG, POAG and exfoliative glaucoma using ELISA technique [62]. This study showed relatively higher aqueous concentration of HGF in glaucoma patients as compared to control subjects. After this, researchers started to associate HGF gene and the role of polymorphisms with PACG condition. First association study for HGF gene was performed by Awadalla and co-workers in Nepalese population [63]. They investigated the association of genetic variations in *HGF* with PACG (N=106) and control (N=204) subjects. Out of 12 SNPs screened, four SNPs, rs5745718 (p=0.002), rs12536657 (p=0.002), rs12540393 (p=0.0006) and rs17427817 (p=0.0006), are significantly associated with PACG in this population. Performing additional studies will be better to understand the role of these polymorphisms in PACG pathogenesis. Later, association of SNPs rs5745718, rs17427817 and rs3735520 was performed by another group in Han Chinese subjects with PACG [64]. This study included 238 PACG patients and 287 controls (age, sex and ethnic matched). This study results into the significant association of rs5745718 and rs17427817 with PACG in Han Chinese population. For SNP rs5745718, CC genotype and C allele were associated with reduced risk of PACG, and for rs17427817, GG genotype and G allele were associated with decreased susceptibility to PACG. However, the other SNP rs3735520 showed no association in this population. Performing further studies with larger sample size and in different ethnic backgrounds will be better to understand the difference in association of the genes with PACG susceptibility and also to understand the functional implications of these SNPs.

21.9.1.3 Serine Protease 56 Gene (*PRSS56*)

Fellow PACG eyes often show some characteristics like shallow anterior chamber depth (ACD), thick lens and shorter axial length (AL) which predispose them to angle closure. Various studies have shown a heritability of ACD, and there are some common genetic determinants shared between ACD, axial length and anterior angle distance. In addition, hyperopia is also a common refractive error linked with shorter axial length. Therefore, genes that control and regulate the growth of axial length might be good candidates for PACG and high hyperopia too.

Serine protease 56 (*PRSS56*) is a serine protease that has been implicated in human eye development. Mutations in PRSS56 gene have been associated in some cases of autosomal recessive posterior microphthalmia and nanophthalmia in humans [65, 66]. In a subsequent study by Jiang

et al., PRSS56 gene variants were evaluated in patients with either PACG (N=189) or high hyperopia (N=110) and in 262 normal controls in Chinese population [67]. This study suggests that variations in *PRSS56* may be associated with PACG and high hyperopia too indicating possible implication of this gene in PACG and hyperopia.

21.9.1.4 CHX10 and Membrane-Type Frizzle-Related Protein Gene (MFRP)

As we know, PACG eyes have small ocular dimensions suggesting the involvement of developmental genes that regulate the ocular dimensions, size and structural remodelling of connective tissues.

CHX10 is a homeobox-containing transcription factor expressed in the retina of the eye. Mutations in this retinal homeobox gene CHX10 have been associated with microphthalmia, characterized by a small eye, and other ocular abnormalities [68]. Membrane-type frizzle-related protein (*MFRP*) gene is a tissue remodelling gene considered as a putative susceptibility gene for extreme hyperopia and nanophthalmos. MFRP is predominantly expressed in the retinal pigment epithelium (RPE) and ciliary epithelial cells of the eye [69, 70]. Therefore, in a study by Aung et al., both *CHX10* and *MFRP* were screened in Chinese subjects for their possible role in PACG patients (N=108) with small ocular dimensions, i.e. axial length less than 22.5 mm [71]. However, results of this study did not support a significant role of mutations in these two genes with PACG as most of the variations are not pathogenic.

Subsequently, another case-control pilot study was conducted in Taiwanese population to evaluate the possible genetic association between acute PACG (N=63) and sequence variants of *MFRP* [72]. Sequence variants were detected by using polymerase chain reaction (PCR) and direct sequencing for exons 4,5 and 10 of MFRP gene, which are already associated with extreme hyperopia condition which is one the factor for PACG [73, 74]. Three variants rs3814762 (A/G, located on exon 4), rs36015759 (T/C, exon 5) and rs2510143 (C/T, exon 5) identified in this pilot study were not associated with acute angle-closure glaucoma.

Later, by another group, the association between primary angle closure (PAC) and the genetic variant rs3814762 of *MFRP* and rs1043618 of *HSP70* (70 kilodalton heat shock protein, see Sect. 21.9.1.5) genes was investigated in a Han Chinese population [41]. PAC is the early stage of PACG, and also it is believed that the progression from primary angle-closure suspect (PACS) stage to PAC is regulated by a tissue remodelling pathway. This study showed suggestive or nominal association of *MFRP* rs3814762 with PAC.

21.9.1.5 Heat Shock Protein 70 Kilodalton Gene (HSP70)

HSP70 is a ubiquitously expressed protein which helps to protect cells from stress. Under stress conditions like hypoxia or any injury, levels of these HSPs increased to protect against the stress, while underexpression leads to incompetent response and results in loss of neuroprotection. Previously, Tezel et al. showed that HSPs boost up the neurodegenerative functions of the immune system in retinal ganglion cells (RGCs) and glial cells, thus facilitating the glaucomatous related changes such as optic nerve damage and neurodegeneration [75]. Also, studies are there showing that the glial cells become activated in glaucomatous eyes, and there is an increased expression of HSPs in these eyes which acts as an immunostimulatory signal leading to break in immune tolerance [75, 76]. Although HSP70 is not directly involved in tissue remodelling process, HSP70 contributes to PACG by affecting the expression of *MMP-9* which contributes in tissue remodelling pathway (see Sect. 21.9.1.1). HSP70 performs this by activating nuclear factor kappa B (NF-kappaB) and activating protein 1 (AP-1) which in turn leads to the activation of MMP-9 transcription [77]. Because of underexpression of HSP, the activity of MMP-9 may be downregulated in acute PACG resulting in shorter axial length during ocular growth and development by the process of extracellular matrix (ECM) remodelling [38]. HSP70 SNP

rs1043618 was thought to be associated in Han Chinese population with primary angle closure (PAC), but this study showed only marginal association [41].

In a separate study, involvement of stress-regulating genes, endothelial nitric oxide synthase (eNOS, see Sect. 21.9.1.6) and HSP70 with POAG and primary closed angle glaucoma (PCAG) was investigated in Pakistani population [78]. SNP rs1043618 (HSP70 G+190C) maps to the 5′ untranslated region (UTR) of *HSP70*.In general, UTRs play a vital role in the post-transcriptional regulation of gene expression [79]. There was no association between SNP rs1043618 and POAG, while highly significant association ($p<0.001$) was observed for PCAG cases in Pakistani cohort.

21.9.1.6 Endothelial Nitric Oxide Synthase (eNOS) Gene

There are number of studies there illustrating the role of eNOS in health and disease. Fotula and colleagues explained and well discussed the characterization of human endothelial nitric oxide synthase promoter and expression in vascular endothelium [80]. The vascular endothelium is an active organ and plays an elementary role in maintaining the vascular tone by releasing various biochemical factors that modulate the contractile and relaxatory behaviour of the underlying vascular smooth muscle, regulation of inflammation, platelet aggregation, etc. [81]. It regulates these processes by activating eNOS which is responsible for nitric oxide (NO) production, and reduced activity of NO leads to endothelial dysfunction. There are lines of evidence that exist suggesting that vascular dysregulation predisposes to the development of glaucoma with compromised blood supply to the optic nerve head which has been implicated in optic nerve damage. Several isoforms of NO are found in the ocular tissues, including the retina, and are synthesized in the vascular endothelium through the action of endothelial nitric oxide synthase on the substrate L-arginine [82]. Over-production of NO results into production of free radicals, peroxynitrite, NO_2 and nitrite which induces various pathophysiological actions, such as optic nerve degeneration and posterior retinal degeneration lesion, leading to glaucomatous condition and many other eye diseases [83, 84]. Nathanson and co-workers showed that there are abnormalities that exist in NO or NO-containing cells in case of POAG which may be related to glaucoma [85].

A study by Neufeld and group demonstrated the increased amounts of three isoforms of nitric oxide synthase (NOS) in the histological sections of optic nerve heads of glaucoma patients that may be locally neurodestructive to the retinal ganglion cell (RGC) axons, thus supporting the hypothesis of optic nerve damage due to eNOS overexpression [86].

Reports are there showing that NO formed by eNOS regulates ocular haemodynamics. It has been shown that eNOS is involved in the pathogenesis of cardiovascular diseases and various neurodegenerative disorders such as glaucoma and diabetic retinopathy [87]. Ayub et al. hypothesized that there is an impaired ocular blood flow which in turn somewhat related to altered NO and eNOS expression that might lead to closure of chamber angle in primary closed angle glaucoma (PCAG) patients [78]. Therefore, they determined the association of a 27 base-pair (27 bp) variable number tandem repeat (VNTR) polymorphism present in the intron 4 of *eNOS* with PCAG and POAG subjects. This polymorphism is believed to affect NO production and thus involved in vascular dysregulation [88]. This study results into association of intron 4 polymorphism with both the study groups, i.e. PCAG and POAG (both $p<0.01$), suggesting association of the disease with increased activity of NOS.

Following this, a haplotype-based case-control study investigated the association of eNOS gene polymorphisms with PCAG ($N=88$) and POAG ($N=102$) in Han Chinese population [89]. Genotyping was done for five SNPs (rs743507, rs3793342, rs11771443, rs7830, rs3918188) using TaqMan assay. This study showed significantly increased frequency of CT haplotype established by rs3793342 and

rs11771443 for POAG patients than controls. However, the study did not find any association between PCAG and *eNOS*. This was in contrast to previous study by Ayub and co-workers may be because of difference in ethnicities.

Recently, Awadalla and colleagues also confirm the association of *eNOS* polymorphisms with PACG pathogenesis in Nepalese and Australian cohort [90]. This study includes 235 PACG patients (106 Nepalese and 129 - Australian) and 492 controls. They found significant association of *eNOS* polymorphism (rs3793342, T allele, p=0.003) with PACG in Australian cohort but not in Nepalese may be due to insufficient power of the study. Later, in another study possible association of *eNOS* variants (rs3793342 and rs11771443) was checked with PAC (N=232) subjects instead of PACG and control subjects (N=306) in Han Chinese [91]. They hypothesized that *eNOS* might contribute to PACG by affecting biometric features such as AL, ACD and dioptre of spherical power (DS). While the two SNPs were not associated with PAC, however, *eNOS* rs11771443 was associated with deeper ACD. Based on these results, they conjectured that the variant rs11771443 might result in an increased ACD by the effect of increased production of NO in anterior segment endothelia which results into ciliary muscle relaxation and thus affecting ACD. In brief, we can say that the association between genetic variants of *eNOS* and PACG is inconsistent and differs between the populations, and their role in PACG pathogenesis needs further studies.

21.9.1.7 5,10-Methylenetetrahydrofolate Reductase Gene (MTHFR)

Despite the heterogeneous nature of glaucoma, clinically, any variant of glaucoma finally results in RGCs' death, and many studies support the apoptotic death of RGCs in glaucoma [92–94]. Increased homocysteine levels (i.e. hyperhomocysteinemia, hypHcy) have been reported to promote RGC apoptosis [95] because of reduced activity of an enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR). MTHFR is involved in the metabolism of homocysteine (Hcy) and folate. The levels of Hcy have been observed to be affected by C677T polymorphism (rs1801133) in the *MTHFR* with reduced MTHFR enzyme activity [96, 97]. This polymorphism results in alanine to valine change at position 222 of the enzyme. Hcy has shown to induce changes in the ECM, vascular injuries and damage to ganglion cell layer neurons by apoptosis [95, 98–100].

In addition, several studies have reported the elevated levels of Hcy in many forms of glaucoma [101–103]. But with regard to angle-closure glaucoma, the first study was conducted by Michael and colleagues in Pakistani population [104]. This was a prospective study which includes 150 patients (90 POAG and 60 PCAG) and 70 controls. Genotyping was done by using PCR-RFLP method. This study concluded that the *MTHFR* C677T showed association with PCAG group but not with POAG patients and thus suggesting C677T as a risk factor for PCAG patients of Pakistani origin. Also, *MTHFR* polymorphism might play an essential role in remodelling of trabecular meshwork (TM) and connective tissue of anterior segment in the development of chronic PCAG. However, no statistically significant associations were observed between *MTHFR* and PACG subjects in Nepalese and Australian population [105]. Ethnicity seems to play an essential role in case of association studies in different populations. Later, study by Shi et al. also showed no differences in genotype and allele frequencies between the PAC and control group [41]. Recently, association of *MTHFR* C677T with PACG and POAG subjects was conducted in North Indian population [106]. This study also concludes that *MTHFR* C677T SNP shows susceptibility to POAG but not with PACG in North Indian cohort.

21.9.1.8 Calcitonin Receptor-Like Receptor (*CALCRL*)

A transgenic mouse model for acute angle-closure glaucoma (ACG) has been created by Ittner and colleagues [107]. In this study,

they observed acute and transient rise in IOP, a characteristic feature in humans for acute angle-closure glaucoma, in CLR (calcitonin receptor-like receptor, or *CALCRL*) transgenic mice. Overexpression of *CALCRL* (adrenomedullin receptor) in the papillary sphincter muscle resulted in papillary palsy and angle closure in CLR transgenic mice. Adrenomedullin (AM) is a smooth muscle-relaxing polypeptide (52-amino acid residue) that is produced and secreted by various types of cells and tissues including the eye [108]. AM has been reported to express in the iris-ciliary body and controlling the IOP through specific AM receptors [109]. AM exerts its actions through specific receptors that consist of CALCRL and receptor activity-modifying protein 2 (RAMP2) heterodimers which were identified in human pupillary sphincter muscle [107]. Therefore, dysregulation of AM action in the pupillary sphincter muscle causes chronic relaxation of sphincter muscle resulting into obstruction of aqueous outflow and, hence, contributing in the development of angle-closure glaucoma.

Cao et al. conducted a study to know whether genetic variations in *CALCRL* have some role in PACG pathogenesis [110]. For this, association analysis was performed in southern Chinese population between variants of *CALCRL* and two patient groups (N=207), acute PACG (APACG) and chronic PACG (CPACG) along with unaffected controls (N=205). This study results in a nominal association of *CALCRL* SNP rs1157699 with APACG patients not with CPACG. This is followed by another study performed in two different ethnicities, i.e. Australian Caucasians and in Nepalese [105]. This group investigated the association of *CALCRL* SNPs (rs2063505, rs840617, rs13411274, rs6759535, rs3821183, rs9288141, rs3771073, rs7591567 and rs6706141) with PACG. But this study reported no association in Nepalese cohort.

Besides the genetic studies discussed in above sections, there were some genetic polymorphisms thought to be associated with PACG in other ethnic populations. But those SNPs did not show significant association as mentioned below (see Table 21.1).

Table 21.1 List of genetic polymorphisms not to be associated with PACG

Gene	Polymorphism	Population	Reference
NT4	A88V	Indian	Rao et al. [111]
ApoE	All three alleles ε2, ε3, ε4	Saudi	Al-Dabbagh et al. [112]
IL-1	−889C/T	Chinese	How et al. [113]
	+3953C/T		
	−511C/T		
LOXL1	rs2165241	Indian	Chakrabarti et al. [114]
	rs1048661		
	rs3825942		

NT4 neutrophic factor, *ApoE* apolipoprotein E (epsilon 2, 3, 4), *IL-1* interleukin-1, *LOXL1* lysyl oxidase

21.9.2 Genome-Wide Association Studies (GWAS)

GWAS proves to be a major tool for investigating the genetic architecture of complex disorders. It not only facilitates the identification of genetic variants responsible for a disease but also helps to understand how selective pressures and natural selection have affected the human genome [115]. After the discovery of associated variation for a disease or trait, it is checked that this bona fide association also affects other populations of different genetic ancestries.

21.9.2.1 PLEKHA7, COL11A1, PCMTD1-ST18 genes

Several association studies for PACG were conducted in different ethnic populations, but still individual susceptibility gene or defined locus for PACG is still unknown. The first GWAS study for PACG conducted by Vithana et al. published in *Nature* genetics includes two stages [116]. The first discovery stage included 11,854 PACG cases and 9608 controls, recruited from five different ethnic groups (Singapore, Hong Kong, India, Malaysia and Vietnam). The second is replication stage which comprised an additional 1917 PACG cases and 8943 controls across six ethnic groups (two places in China and one each in Singapore, India, Saudi Arabia and the UK). This study found significant association of three susceptibility loci rs11024102 in PLEKHA7 gene, rs3753841 in COL11A1 gene

and rs1015213 located between PCMTD1 and ST18 gene as genetic risk factors for PACG.

SNP rs11024102 lies in the PLEKHA7 gene, which encodes a pleckstrin homology domain-containing protein 7 involved in the stability and maintenance of adherens junctions (AJ) between the cells [117, 118]. These tight junctions and adherens junctions provide a barrier to fluid leakage and have a vital role in structures such as the ciliary body, iris, aqueous humour outflow system and choroid [119]. Expression profile analysis and localization study of *PLEKHA7* in the ocular tissues revealed its specific localization in regions particularly relevant to PACG such as the ciliary body, iris and choroid. Other sites include the blood-aqueous barrier (BAB) structures, posterior iris epithelium, non-pigmented ciliary epithelium, iris and ciliary body microvasculature [120].

The second SNP rs3753841 lies within the coding region of COL11A1 gene causing a non-synonymous (c.3968C >T) change that leads to substitution of proline to leucine at 1323 amino acid position. This gene encodes one of the two α chains of type XI collagen. Pathogenic mutations in *COL11A1* have been associated with Marshall syndrome, Stickler syndrome or type II (STL2) or Stickler-like syndrome [121]. The third associated marker rs1015213 on chromosome 8q is located within an intergenic region 120 kb upstream of PCMTD1 and 130 kb downstream of ST18 [116]. *PCMTD1* encodes protein-L-isoaspartate O-methyltransferase domain-containing protein 1, whose function has not been well characterized. The gene ST18 (suppression of tumorigenicity 18) encodes a zinc finger DNA-binding protein and has been known as a breast cancer tumour suppressor gene [122]. Recently, few studies also have shown it as a mediator of apoptosis and inflammation [123].

Subsequently, Duvesh et al. replicated this study in South Indian population to determine the susceptibility of these loci with subtypes of primary angle-closure patients [124]. This study includes three case groups: primary angle-closure/primary angle-closure glaucoma (PAC/PACG, N=180), primary angle-closure suspect (PACS, N=171) and a combined any-angle closure group consisting of PACS and PAC/PACG cases. Control group included 411 individuals (age and ethnic matched). Genotyping was performed using the TaqMan allelic discrimination assay. This study showed that SNP rs1015213 was associated significantly with PAC/PACG group (p=0.002) and any closure group (p=0.003), hence confirming prior reports. However, no significant genetic association was seen in PACS subjects (P=0.052). In addition, there was no significant association (p>0.05) observed for other SNPs rs3753841 (*COL11A1*) and rs11024102 (*PLEKHA7*) with angle-closure phenotypes. The non-significant associations of these two SNPs can be overcome by increasing the sample size in future studies to confirm the importance of these genes in the pathogenesis of glaucoma.

Similarly, Awadalla and colleagues also investigated the role of these variants in two different cohorts (Nepal and Australia) with PACG subjects [125]. In the Nepalese cohort, both rs1015213 (p=0.014; odds ratio 2.35) and rs11024102 (p=0.039; OR 1.43) showed significant association with disease development. While in the Australian cohort, SNP rs3753841 was found to be significantly associated with PACG.

Three newly discovered PACG loci through GWAS were analysed in different ethnic populations for their susceptibility. Apart from the association of genetic locus with the particular disease, researchers are also interested to look into association of genes with various characteristic features of disease phenotype (e.g. for PACG various features such as axial length (AL), anterior chamber depth (ACD), etc.). Nongpiur and co-workers evaluated the association of SNPs rs11024102 in *PLEKHA7*, rs3753841 in *COL11A1* and rs1015213 in *PCMTD1-ST18* with biometric parameters ACD and AL in Singaporean and Beijing cohort [126]. But this study showed lack of association between PACG susceptibility loci with ACD and AL. A study by Shi et al. conducted in Jiangsu, China, also observed similar results [127]. Their results did not support the association of three loci (rs11024102, rs3753841 and rs1015213) with PAC group and ocular biometry (AL,

ACD and dioptre of spherical power, DS). However, Chen et al. found mild association of SNPs rs11024102 in *PLEKHA7* and rs3753841 in *COL11A1* with increased risk for PAC/PACG in Han Chinese population [128]. In addition, they also genotyped some other SNPs (by either tagging or coding SNPs with minor allele frequency >10% in Han Chinese populations-HapMap database) in the same genes: rs1676486, rs12138977, rs2126642 and rs2622848 in *COL11A1* as well as rs216489, rs1027617, rs366590, rs11024060, rs6486330 and rs11024097 in *PLEKHA7*. Apart from rs11024102 and rs3753841, other two SNPs rs1676486 and rs216489 showed stronger association with PAC/PACG.

Geneticists are also interested to explore the involvement of SNPs in disease progression and its correlation with particular phenotype. Wei and colleagues performed a genotype-phenotype correlation analysis to investigate the association of PACG polymorphisms discovered in a study by Vithana et al. with phenotype of PACG patients in terms of disease severity or progression [129]. However, investigators did not observe any significant association between proportion of VF progression and blindness with PACG loci.

In order to address the discrepancies in genetic association studies because of small genetic effects and limited power of the study, researchers are performing meta-analysis [130]. Meta-analysis is a statistical tool that combines results from the original and successive replication studies and helps to resolve the inconsistency in the association studies' results [131]. Recently, a meta-analysis study was carried out by Bai et al. to determine the association of rs3753841 with PACG [132]. This study showed that *COL11A1* may confer higher susceptibility to PACG. Following this, another study published recently by Shuai and group evaluated SNP rs11024102 and rs3753841 for the association with PACG [133]. This study suggests both rs11024102 and rs3753841 are associated in Asian population, while rs3753841 in *COL11A1* showed significant association with PACG only in Caucasian population.

21.9.2.2 ABCC5 Gene

Recently, another GWAS study was conducted by Nongpiur and colleagues in the individuals of Asian descent [134]. This study was focussed on anterior chamber depth (ACD), a risk factor for PACG patients to identify gene variants influencing ACD. A total of 5308 population-based individuals was recruited in the first stage of the study. Subsequently, identified variant rs1401999 was tested for association in a new set of samples consisting of 4276 PACG cases and 18,801 controls. They reported SNP rs1401999 in ABCC5 gene that was significantly associated with ACD together with an increased risk of having PACG. *ABCC5* in humans encodes multidrug resistance-associated protein 5 (MRP5), a member of ATP-binding cassette (ABC) transporters. It is expressed in several ocular tissues such as cornea [135], retina and retinal pigment epithelium [136]. In this study, expression of *ABCC5* was observed in the anterior segment structures such as the iris, ciliary body and lens. However, its exact role is still not clear. The function of ABCC5 has been shown to participate in tissue defence and cellular signal transduction by cellular extrusion of xenobiotics, toxicants and cGMP [137–139]. In view of the fact that shallow anterior chambers make angle region more crowded and thus increasing the risk of developing PACG. Therefore, screening the genetic variants for ACD in PACG patients will shed light in some way to understand the influence and contributions of these variants in PACG pathogenesis.

21.10 Other Genes

There are a number of other genes which are thought to be having a role in PACG pathogenesis; however genetic screening revealed no association. *Myocilin* (*MYOC*) is one of the very well-known candidate genes for POAG, but little is known with reference to association with PACG. Phenotypically, some similarities exist between POAG and chronic PACG and

therefore, Aung et al conducted a study in Chinese subjects and screened 106 patients with chronic PACG [140]. Sequence variations were identified by bidirectional sequencing. But this study does not support the role *MYOC* mutations in the pathogenesis of chronic PACG. Additionally, another study in the Middle Eastern patients was conducted in which PACG patients (N=29) were evaluated for nuclear gene mutations known to be associated with glaucoma and any mitochondrial abnormalities [141]. There were no novel or reported mutations found in the *MYOC*, optineurin gene (*OPTN*), WD repeat domain 36 gene (*WDR36*), cytochrome P450 family 1 subfamily B polypeptide 1 gene (*CYP1B1*), optic atrophy 1 gene (*OPA1*) and optic atrophy 3 gene (*OPA3*). In addition, mitochondria-related abnormalities were also minimal in PACG subjects.

21.11 Summary

In summary, genetic research offers a framework to unravel the genetic basis of complex diseases. There are plenty of researches going on worldwide to understand the intricate genetics of PACG, and significant progress has been achieved till now. Several genetic approaches including genome-wide association studies prove to be very useful to rule out the underlying genetics in terms of finding susceptible loci in the genome responsible for angle-closure glaucoma. Further studies on these novel loci revealing their exact role in disease mechanism will hopefully underpin the complex genetic behaviour of PACG in the near future.

Compliance with Ethical Requirements Roopam Duvesh, Rengaraj Venkatesh, Srinivasan Kavitha, Pradeep Y. Ramulu, Subbiah Ramasamy Krishnadas and Periasamy Sundaresan declare that they have no conflict of interest.

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

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Genotype-Phenotype Correlation for POAG in the Middle East and Other Populations

22

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Abstract

POAG is a genetically complex trait with a substantial fraction exhibiting a significant heritability. Less than 10% of POAG cases in the general population are caused by specific gene mutations and the remaining cases are polygenic. Epidemiological studies suggest that by 2020 the prevalence of primary open angle glaucoma (POAG) is estimated to increase to 76.0 million, and to 111.8 million by 2040 globally due to the population aging. The prevalence of POAG is the highest among those of African descent, followed by Asians, and the lowest in Europeans. Quantitative traits related to POAG pathogenesis such as intra-ocular pressure (IOP), vertical cup/disc ratio (VCDR), optic disc area, and central corneal thickness (CCT) are highly heritable, and likely to be influenced at least in part by genes and show substantial variation in human populations. Recent genome-wide association studies (GWAS) have identified several single nucleotide polymorphisms (SNPs) at different loci including CAV1/CAV2, TMCO1,CDKN2B-AS1, CDC7-TGFBR3, SIX1/SIX6, GAS7 and ATOH7 to be associated with POAG and its related quantitative traits (endophenotypes). The chapter provides a brief overview on the different GWAS and SNP association studies and their correlation with various clinical parameters important for POAG in the population worldwide, including the Middle East.

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22.1 Introduction

Glaucoma is a leading cause of irreversible blindness affecting approximately over 60 million people globally [1]. It is a heterogeneous group of optic neuropathy that is chronic and progressive, associated with loss of retinal ganglion cells and axons resulting in characteristic cupping of the optic nerve head or degeneration of the optic nerve and visual field defects [2]. Primary open-angle glaucoma (POAG) is the most common form of glaucoma defined by an open and normal appearing anterior iridocorneal chamber angle [2]. POAG can occur with or without increased intraocular pressure (IOP), the latter being referred to as normal-tension glaucoma (NTG).

Although there are many postulated mechanisms of retinal ganglion cell damage, the exact etiology of POAG still remains obscure. The well-recognized risk factors associated with POAG include elevated IOP, age, family history, gender, ethnicity, central corneal thickness, and myopia. A recent large prospective study indicated that POAG with early paracentral visual field loss displays distinct as well as common risk factor profiles as compared to those with peripheral vision loss [3]. Raised IOP is the most important and the only modifiable risk factor for the development of POAG. Several large population-based studies in the past have confirmed that reduction of IOP reduces progression of glaucoma in patients with or without elevated IOP [4–8].

Similarly, the prevalence of glaucoma increases with the increasing age in all ethnicities (Europeans, Blacks, and Hispanics) as shown by the meta-analysis data from the Eye Diseases Prevalence Research Group [7, 9]. Age was also reported to be an important risk factor for the development of POAG in patients with ocular hypertension in both the Ocular Hypertension Treatment Study [5] and the European Glaucoma Prevention Study [8]. Family history has been consistently shown to be a risk factor for glaucoma [7, 10–12]. A positive family history of POAG significantly increases the odds (varying from 5 to 10 times) for the development of POAG [13]. In the Melbourne [14] and Rotterdam studies [11], there was a trend toward increased risk for POAG in males which were absent in the Barbados Eye Study [7] and the Beaver Dam Eye Study [10]. Similarly, the Eye Disease Prevalence Research Group [9] found no gender-related difference between the prevalence of glaucoma among the white, black, and Hispanic populations. However, a recent systematic review of 3497 POAG cases out of 146 882 participants with gender-specific data showed that the age-adjusted prevalence is higher in men compared to women and that this finding remains consistent across all ethnic groups provides a very strong evidence for the association of POAG with gender [15]. Several studies have shown POAG to be more prevalent with rapid and severe disease progression in people of African-Caribbean as compared to European descent, Hispanics, and Asians [9, 16]. Central corneal thickness (CCT) has also been shown to be a risk factor for POAG particularly in the ocular hypertension patients [5, 17]. Although the precise mechanism(s) are still unclear, but this may be in part due to the effect of corneal thickness on IOP measurement, increased susceptibility to optic nerve damage [18, 19]. In addition, studies have shown that individuals with thicker corneas are less responsive to topical ocular hypotensive medications [20]. Myopia can also result in increased susceptibility of myopic nerves to glaucomatous damage and is considered to be a risk factor for POAG [21]. Moderate to high myopia (spherical equivalent of −3 D refractive error or greater) conferred two- to threefold increased risk in the

Australian [22], US Caucasian [23], and the Chinese population [24]. Other predisposing factors for POAG include adult-onset diabetes and hypertension. Although there are conflicting reports regarding the risk of POAG in individuals with diabetes [25, 26], however, a recent systematic review and meta-analysis of 13 studies which included six population-based cohorts and seven case-control studies showed increased risk of POAG (relative risk of 1.4 and 1.49, respectively) in individuals with diabetes [27]. Multiple epidemiological studies have also reported a role of hypertension as a risk factor for POAG [22, 28]. Treatment of hypertensive patients with beta-blockers results in nocturnal hypotension and is a potential risk factor for glaucomatous optic neuropathy [29]. The mechanism(s) by which hypertension induces optic nerve damage are still unclear.

POAG is a genetically complex trait with a substantial fraction exhibiting a significant heritability. Genetic linkage studies of large affected families have so far identified at least 20 chromosomal loci (GLC1A-P) that are linked to POAG. The causative genes that are capable of causing POAG with minimal influence from other gene (s) or the environment and have been consistently implicated so far include myocilin (*MYOC*), optineurin (*OPTN*), WD repeat domain 36 (*WDR36*), ankyrin repeat and SOCS-box containing 10 (*ASB10*), cytochrome P450 family 1, subtype B, polypeptide 1 (*CYP1B1*), and neurotrophin 4 (*NTF4*) as reviewed elsewhere [30, 31]. Twin studies and family-based studies have discovered a number of genes. However, these disease-causing genes account for $<$10% of POAG cases in the general population. It is therefore likely that the hereditary aspect of many of the remaining cases of POAG is due to the combined effects of several genes (polygenic) and that gene-environment interactions are important. Quantitative traits related to POAG pathogenesis such as IOP, vertical cup-to-disc ratio (VCDR), and CCT [10, 32, 33] are highly heritable, likely to be influenced at least in part by genes and show substantial variation in human populations. Recent advances in genomic technologies and genome-wide association studies (GWAS) have greatly accelerated the discovery and understanding of genes and genomic regions associated with POAG and influencing the quantitative traits related to POAG pathogenesis which will be the main focus of this chapter.

22.2 Epidemiology of POAG

Recent epidemiological studies suggest that in 2013, almost 64.3 million people (aged between 40 and 80 years) were affected by glaucoma worldwide, and by 2020 this number is expected to increase to 76.0 million and 111.8 million by 2040 due to population aging [34]. POAG is the most common form of glaucoma accounting for a major three-quarters (74%) of all glaucoma cases [1]. Another recent meta-analysis estimated the global number of POAG cases in 2015 at 57.5 million, rising to 65.5 million by 2020 [15]. Almost half (47%) of these people will reside in Asia while 24% will be European [1]. The risk and subtypes of glaucoma are known to vary among races and countries [35]. A meta-analysis conducted by The Eye Disease Prevalence Research Group showed that in the USA, Blacks have a higher POAG prevalence than Whites. The overall prevalence of POAG in individuals $\geq$40 years old is 1.86%, affecting 1.57 million Whites and 398,000 Black individuals. In 2020, due to the rapidly aging population, it is estimated that this number will grow to 3.36 million [9]. In every age group, there was a higher prevalence in individuals of African descent compared with European-derived individuals [36]. Similarly, a recent meta-analysis of 81 studies including 37 countries, 216,214 participants, and 5266 POAG cases reported that the Black populations had the highest POAG prevalence of 5.2% (95% credible interval (CrI) 3.7%, 7.2%) at 60 years, rising to 12.2% (95% CrI 8.9–16.6%) at 80 years. The increase in POAG prevalence per decade of age was found to be highest among the Hispanics (2.31, 95% CrI 2.12, 2.52) and White populations (1.99, 95% CrI 1.86, 2.12) and lowest in East and South Asians (1.48, 95%

CrI 1.39, 1.57; 1.56, 95% CrI 1.31, 1.88, respectively). In addition, men were more likely to have POAG than women (1.30, 95% CrI 1.22, 1.41). It is clearly evident that individuals of African descent are associated with increased risk (estimated incidence is 2–5 times higher) of developing glaucoma compared with individuals of European descent. The reasons for this increased risk of glaucoma among individuals of African descent are still not clear. The Barbados Eye Study reported a prevalence of 7% in the Africans suggesting an influence of ancestral factors [7]. Several other factors could be involved such as physiologic differences in the optic disc or thinner corneas, environmental factors, or genetic. Social differences including less access to health care may also be influential [25].

22.3 Asian Perspective

Asians account for almost half of the glaucoma population worldwide [1]. Multiple studies have evaluated the prevalence of POAG among different parts (East, South, and Southeast) of Asia [16, 37–41]. A recent population-based study has reported a comprehensive analysis of prevalence of glaucoma in Asians [42]. The study reported a prevalence of 2.02% POAG in East Asia (China, Japan, and South Korea), 2.36% in South Asia (India, Bangladesh, and Sri Lanka), and 2.1% in Southeast Asia (Myanmar, Thailand, and Singapore). The prevalence of POAG was found to be almost similar in different parts of Asia with a mean prevalence of 2.2%. In addition, POAG prevalence was higher among the urban population than the rural population. This pattern of urban to rural distribution was similar across Asia. The calculated average prevalence of POAG from all parts of Asia was found to be 2.63% from urban regions as compared to 2.04% rural regions (statistically nonsignificant, $p = 0.159$). These findings are well supported by another recent systematic review and meta-analysis of 23 population-based studies of 1318 POAG cases (aged 40–80 years) in Asia. The study reported an overall glaucoma prevalence of 3.54% (95% CrI 1.83–6.28) in 2013, with POAG accounting for 2.34% (95% CrI 0.96–4.55) [43]. It also estimated that South Central Asia will record the highest increase in number of glaucoma individuals from 17.06 million to 32.90 million from 2013 to 2040 compared with other Asian subregions highlighting an increased disease burden across Asia and an urgent need for sustainable public health strategies to combat glaucoma in Asia [43].

As compared to other populations worldwide, the prevalence of POAG is the highest in those of African descent, followed by Asian patients, and the lowest in European descent [7, 9, 42]. Differences in anatomy, pigmentation, and/or genetic susceptibility may at least in part account for these apparent ethnic variations [35]. Many studies have proposed numerous factors affecting the prevalence of POAG in Asia. The Chennai Glaucoma Study suggested inherent socioeconomic and lifestyle differences between the urban and rural populations indicating that urban living settings predisposes to higher rates of glaucoma [44]. The study from Harbin, China, showed education level, family history, and systemic hypertension as risk factors for POAG along with increased age and elevated IOP [45]. Some study also supports the idea that lower rates are associated with colder climates [46]. The Singapore Malay Eye Study reported low diastolic blood pressure, low mean ocular pressure, and low diastolic perfusion pressure as independent risk factors for open-angle glaucoma [47]. Other studies have reported history of thyroid disease and male gender as a risk factor for POAG [16, 41, 48].

22.4 Genotype-Phenotype Association in POAG

Association studies using candidate-gene approach and GWASs have been particularly useful tools in identifying genetic factors, each of which may have a relatively small effect but contributes to a large number of cases. Unlike candidate gene approach, GWAS is an unbiased (without bias to known protein functionality

gene) genome-wide approach that compares the genotypic profile of single nucleotide polymorphisms (SNPs) throughout the genome in cases (affected) and controls (unaffected) thus identifying genomic region(s) associated with a disease or trait of interest. A large population sample required in GWASs to achieve a genome-wide statistical significance (P value of less than 5×10^{-8}) has been greatly facilitated by the formation of International Consortia. However, since GWAS can rarely identify functional or causal variant(s), further in-depth genotyping and functional testing in addition to replication studies in independent cohorts of different population groups is considered a standard requirement to conclusively validate genes or genomic regions identified from GWAS. Using this powerful approach (GWAS), recent genetic studies have identified genes or genetic variants with modest effect to be associated with POAG and related quantitative traits (Table 22.1). These studies have provided better insights into the genetic basis of POAG and improved our understanding to the underlying pathophysiology of the disease.

22.5 GWAS and POAG

The first GWAS for POAG was described by Nakano et al. in the Japanese population with patients predominantly having NTG [49]. This was a 2-stage GWAS involving a discovery cohort and a replication cohort. The loci reported are on chromosomes 1, 10, and 12 with the nearest annotated genes *ZP4*, *PLXDC2*, and *TMCT2* (*DKFZp762A217*), respectively. However, none of the SNPs reached genome-wide significance ($P < 5 \times 10^{-8}$) even in the combined analysis and therefore await further evaluation in additional cohorts. Meguro et al. reported the first genome-wide significant ($P = 2.5 \times 10^{-9}$, odds ratio (OR) = 2.80) association for SNP rs3213787 in *SRBD1* in the Japanese NTG population [50]. These results were replicated in two other studies involving a cohort of Japanese NTG and high-tension glaucoma (HTG) [51] and a POAG cohort in the USA [52] but not in the African-Caribbean cohort [53].

GWAS for POAG have successfully identified common variants near *CAV1* and *CAV2* in an Icelandic cohort [54], in *TMCO1* and *CDKN2B-AS1* in an Australian cohort [55], in *CDKN2B-AS1*, *SIX1*/*SIX6*, and 8q22 regions in individuals of European ancestry [56], in *GAS7* and *TMCO1* in US Caucasians [57], and in *CDKN2B-AS1*, *CDC7*/*TGFBR3*, and *FNDC3B* in Asian, African, and European cohorts [58].

The *caveolin* genes have been postulated to influence transforming growth factor-beta (TGF-ß) or nitric oxide signaling pathways involved in POAG pathogenesis. The locus on chromosome 7q31 has been studies in the US Caucasians, Africans, and the Saudi Arabian population with inconsistent results [53, 54, 59–62]. A recent meta-analysis of five studies, including 5774 POAG cases and 40,598 controls, suggested that SNP rs4236601 is associated with increased risk for POAG in Caucasian and Asian populations but not in African and Saudi populations [63]. Australian GWAS identified two loci *TMCO1* (1q24) and *CDKN2B-AS1* (9p21) to be associated with advanced glaucoma. The association of *TMCO1* locus with POAG has been replicated in another GWAS for a Caucasian cohort [57] and associated with increase in IOP as well [57, 64], and the carriers of risk alleles for SNP rs4656461 have been reported to be associated with younger age at diagnosis [65]. TMCO1 is highly expressed in the ciliary body, trabecular meshwork, and retina. However, its precise role in POAG pathogenesis is unclear. So far, there are no published reports of association studies at *TMCO1* locus in the middle east population.

Since the identification of association between *CDKN2B*/*CDKN2B-AS1* locus and POAG in the Australian cohort, several GWASs have replicated this association in the US Caucasian [56], Japanese [66–68], Asian, African, and European population [58] providing a strong evidence for the association of this locus with POAG. In addition, many studies have reported positive association of SNPs in *CDKN2B* in several other

Table 22.1 Genes and polymorphisms identified in POAG using genome-wide and candidate gene approaches in the middle east and other population

Studies	Gene/ chromosome	SNP ID	Population[a]	Study type	Study size (POAG/ controls)[a]	OR/Beta, *P* value	Any clinical association[a]
GWAS studies							
Nakano et al. 2009 [49]	*PLXDC2 (10p12.31)*	rs7081455	D: Japan	GWAS	D: 1519	OR = 1.49,	–
						$P = 1 \times 10^{-5}$	
	TMTC2 (12q21.31)	rs7961953				OR = 1.37,	–
						$P = 7 \times 10^{-5}$	
	ZP4 (1q43)	rs547984	R: Japan		R: 857	OR = 1.34,	–
						$P = 6 \times 10^{-5}$	
		rs540782				OR = 1.34,	
						$P = 6 \times 10^{-5}$	
		rs693421				OR = 1.35,	
						$P = 4 \times 10^{-5}$	
		rs2499601				OR = 1.33,	
						$P = 9 \times 10^{-5}$	
Meguro et al. 2010 [50]	*SRBD1 (2p21)*	rs3213787	Japanese	GWAS	D: 305	OR = 2.80,	Associated with NPG
	ELOVL5 (6p12.1)	rs735860			R: 355	$P = 2.5 \times 10^{-9}$	
						OR = 1.69,	Associated with NPG
						$P = 4.1 \times 10^{-6}$	
Thorleifsson et al. 2010 [54]	*CAV1/CAV2 (7q31.1)*	rs4236601	D: Iceland	GWAS	D: 36,140	OR = 1.36	Nominal association was observed for increased IOP ($P = 0.034$)
			R1: SW, UK, AU		R1: 4239		
			R2: China			$P = 5 \times 10^{-10}$,	
		rs1052990			R2: 879	OR = 1.32,	
						$P = 1.1 \times 10^{-9}$	–
Burdon et al. 2011 [55]	*CDKN2B-AS (9p21.3)*	rs4977756	AU, NZ	GWAS	D: 590/3956	OR = 1.50,	–
						$P = 4.7 \times 10^{-9}$	
	TMCO1 (1q24)	rs4656461			R: 4148	OR = 1.68,	–
						$P = 6.1 \times 10^{-10}$	

Wiggs et al. 2012 [56]	*CDKN2B-AS (9p21)*	rs2157719	US Caucasian	GWAS	D: 3146/3487	OR = 0.69,	Also associated with NPG. OR = 0.58, P=1.17×10^{-12}
						$P = 1.86 \times 10^{-18}$	
	SIX1/SIX6 (14q23)	rs10483727				OR = 1.32,	–
	8q22	rs284489				$P = 3.87 \times 10^{-11}$	
						OR = 0.62,	Associated with NPG
						$P = 8.88 \times 10^{-10}$	
Osman et al. 2012 [66]	*CDKN2B-AS (9p21)*	rs1063192	Japanese	GWAS	D: 7993	OR = 0.75,	–
						$P = 5.2 \times 10^{-11}$	
	SIX1/SIX6 (14q23)	rs10483727			R: 9014	OR = 0.79,	–
	NCKAP5 (2q21.2)	rs7588567				$P = 9.49 \times 10^{-8}$,	
						OR = 0.85,	–
						$P = 3.89 \times 10^{-7}$,	
Nakano et al. 2012 [67]	*CDKN2B-AS (9p21.3)*	rs7865618	Japanese	GWAS	D: 833/686	OR = 1.78,	Strongly associated with POAG and POAG/NPG, but not with HPG
						$P = 9.0 \times 10^{-11}$	
		rs523096			R: 411/289	OR = 1.76,	
						$P = 1.6 \times 10^{-10}$	
Takamoto et al. 2012 [68]	*CDKN2B (9p21)*	rs523096	Japanese	GWAS	D: 286/557	OR = 2.13,	Associated with NTG
					R: 183/514	$P = 4.96 \times 10^{-11}$	
Chen et al. 2014 [83]	*ABCA1 (9q31.1)*	rs2487032	Asian Southern Chinese	GWAS	D: 1007/1009	OR = 0.69	–
						$P = 1.66 \times 10^{-8}$	
						$OR_R = 0.73$	
						$P_R = 2.79 \times 10^{-9}$	
	PMM2 (16p13.2)	rs3785176			R: 1899/4965	OR = 1.42	–
						$P = 3.18 \times 10^{-6}$	
						$OR_R = 1.30$	
						$P_R = 5.77 \times 10^{-10}$	

(continued)

Table 22.1 (continued)

Studies	Gene/ chromosome	SNP ID	Population[a]	Study type	Study size (POAG/ controls)[a]	OR/Beta, *P* value	Any clinical association[a]
Gharahkhani et al. 2014 [81]	*ABCA1 (9q31.1)*	rs2472493	D: Australian	GWAS	D: 1155/1992	OR = 1.31,	–
			R1: Australian				
	AFAP1 (4p16.1)	rs4619890	R2: US		R1: 932/6862	$P = 2.1 \times 10^{-19}$	
						OR = 1.20,	–
						$P = 7.0 \times 10^{-10}$	
	GMDS (6p25.3)	rs11969985			R2: 2616/2634	OR = 1.31,	–
						$P = 7.7 \times 10^{-10}$	
Li et al. 2015 [58]	*CDKN2B-AS1 (9p21)*	rs2157719	Asian, African and European	GWAS	D: 3504/9746	OR = 0.71	–
						$P = 2.81 \times 10^{-33}$	
	CDC7-TGFBR3 (1p22)	rs1192415				OR = 1.13	Associated with Optical disk, vertical CD ratio
						$P = 1.60 \times 10^{-8}$	
	FNDC3B (3q25.31)	rs4894796			R: 9173/26,780	OR = 0.89	–
						$P = 7.93 \times 10^{-8}$ in Asians only	
van Koolwijk et al. 2012 [57]	*GAS7 (17p13.1)*	rs11656696	D: NL	GWAS	D: 11,972	$P = 1.4 \times 10^{-8}$	Associated with IOP reduction
			R: UK, AU,		R: 7482	SNP:	
			Canada, NZ			$P = 2.4 \times 10^{-2}$	
			SNP: NL, GE				
	TMCO1 (1q24.1)	rs7555523			SNP: 1432	$P = 1.6 \times 10^{-8}$	Associated with IOP increase
						SNP:	
						$P = 9.1 \times 10^{-4}$	
Hysi et al. 2014 [82]	*FNDC3B (3q25.31)*	rs6445055	Asian, European	GWAS	D: 35,296	$P = 4.19 \times 10^{-8}$	All 4 loci associated with IOP
	ABCA1 (9q31.1)	rs2472493			R: 4284/95,560	$P = 2.8 \times 10^{-11}$	
	ABO (9q34.2)	rs8176693				$P = 6.39 \times 10^{-11}$	
	11p11.2	rs747782				$P = 1.04 \times 10^{-11}$	
Chen et al. 2015 [80]	*FAR2 (12p11.22)*	rs4931170	US Caucasian	GWAS	D: 1660	$P = 1.2 \times 10^{-5}$	Associated with IOP
	GGA3 (17q25.1)	rs52809447				$P = 6.7 \times 10^{-5}$	
	PKDREJ (22q13.31)	rs7291444				$P = 7.4 \times 10^{-5}$	

Springelkamp et al. 2015 [84]	*ARHGEF12 (11q23.3)*	rs58073046	D: NL	GWAS	D: 8105	$\beta = 0.44$,	Associated with increasing IOP
						$P = 1.87 \times 10^{-8}$	
						OR = 1.66,	
			R: NL, UK		R: 1125/4117	$P = 2.81 \times 10^{-9}$ (HPG)	
						OR = 1.29,	
						$P = 4.23 \times 10^{-2}$ (NPG)	
Ramdas et al. 2010 and 2011 [75, 76]	*ATOH7 (10q21.3–22.1)*	rs1900004	D: NL	GWAS	D: 7360	$\beta = -0.068$,	Optic disc area (−)/VCDR (−)
						$P = 2.05 \times 10^{-32}$	
	CDC7/TGFBR3 (1p22)	rs1192415				$\beta = 0.064$,	Optic disc area (+)
						$P = 1.82 \times 10^{-27}$	
	CDKN2B (9p21)	rs1063192	R: NL, UK		R: 4455	$\beta = -0.014$,	VCDR (−)
	SIX1	rs10483727				$P = 1.96 \times 10^{-14}$	
						$\beta = 0.012$,	VCDR (+)
	(14q22.3-q23)					$P = 9.30 \times 10^{-11}$	
	SALL1	rs1362756				$\beta = 0.028$,	Optic disc area (+)
	(16q12.1)					$P = 6.48 \times 10^{-8}$	
Macgregor et al. 2010 [85]	*ATOH7*	rs3858145	D: AU	GWAS	D: 1368	$P = 3.4 \times 10^{-10}$	Associated with mean disc area
	(10q21.3–22.1)		R: UK		R: 848		
	RFTN1 (3p24)	rs690037				$P = 1.6 \times 10^{-6}$	Explained 2.1% cup area variation in AU cohort
Khor et al. 2011 [86]	*CARD10 (22q13.1)*	rs9607469	D: Asian	GWAS	D: 4445	$P = 2.73 \times 10^{-12}$	Associated with optic disc area
	ATOH7 (10q21.3–22.1)	rs7916697	R: NL		R: 9326	$P = 2.00 \times 10^{-15}$	Associated with optic disc area in Asians
	CDC7/TGFBR3 (1p22)	rs1192415				$P = 7.57 \times 10^{-17}$	
Iglesias et al. 2014 [87]	*SIX6 (14q23)*	rs33912345 (His141Asn)	D: NL, UK	GWAS	D: 292/1208	$P = 7.74 \times 10^{-7}$	Associated with VCDR and POAG
		rs146737847 (Glu29Lys)	R: NL, UK		R:11,473	$P = 5.0 \times 10^{-3}$	Associated with VCDR

(continued)

Table 22.1 (continued)

Studies	Gene/ chromosome	SNP ID	Population[a]	Study type	Study size (POAG/ controls)[a]	OR/Beta, *P* value	Any clinical association[a]
Vitart et al. 2010 [90]	*COL5A1*	rs1536482	Croatia, Scotland	GWAS	D: 7711	ß = 0.22,	Associated with CCT
	9q34.2					$P = 7.1 \times 10^{-8}$	
	ZNF469	rs12447690			R: 2681	ß = 0.23,	
	16q24.2					$P = 4.4 \times 10^{-9}$	
	AKAP13	rs6496932				ß = 0.13,	
	15q24–25					$P = 1.4 \times 10^{-8}$	
	AVGR8	rs1034200				ß = 0.14,	
	13q12.11					$P = 3.5 \times 10^{-9}$	
Vithana et al. 2011 [91]	*ZNF469*	rs12447690	D1: SG-Malay	GWAS	D1: 3280	β = −5.068,	Associated with CCT
	(16q24)					$P = 1.92 \times 10^{-14}$	
		rs9938149	D2: SG-Chinese		D2: 3400	β = −6.248,	
						$P = 1.63 \times 10^{-16}$	
	COL5A1/RXRA (9q34.2-q34.3)	rs1536478				β = −4.63	
						$P = 3.05 \times 10^{-9}$	
		rs7044529				β = 2.7	
						$P = 1.2 \times 10^{-4}$	
	COL8A2	rs96067				β = −4.799	
	(1p34.2)					$P = 5.40 \times 10^{-13}$	
Ulmer et al. 2012 [92]	*ZNF469*	rs12447690	D: US-Cau	GWAS	D: 1117	β = −5.08,	Associated with CCT
	(16q24)					$P = 0.001$	
	NTM	rs7481514	SNP: US-Cau		SNP:6469	β = −6.89,	Associated with reduced CCT
						$P = 1.03 \times 10^{-5}$	
	(11q25)					OR = 1.28,	and POAG risk in low-tension subset
						$P = 9.9 \times 10^{-4}$	
Candidate gene studies							
Chen et al. 2012 [95]	*2p16.3*	rs1533428	China	SNP	462/577	OR = 2.16,	Associated with late-onset POAG
						$P = 0.00025$	
Kim et al. 2014 [98]	*10p12.31*	rs7098387	Korea	SNP	211/904	OR = 2.0,	Associated with POAG
						$P = 0.00038$	
Fan et al. 2005 [99]	*APOE*	rs429358	Japan	SNP	400/281	OR = 0.4,	APOE4 confers a protective effect against NTG
		rs7412				$P = 0.007$	

Lam et al. 2006 [100]	*19q13.2*	rs429358	China	SNP	400/300	OR = 0.36,	APOE4 confers a protective effect against NTG
		rs7412				$P = 0.008$	
Lake et al. 2004 [101]		rs429358	UK	SNP	155/349	P = ns	None
		rs7412					
Cao et al. 2012 [53]	*ATOH7*	rs7916697	African-Caribbean	SNP	272/165	OR = 0.67,	Interacts with rs1063192 near *CDKN2B* to reduce POAG risk
						$P = 0.0096$	
		rs1900004				OR = 1.02,	None
						$P = 0.9076$	
		rs3858145				OR = 0.98,	None
						$P = 0.9138$	
Mabuchi et al. 2012 [70]		rs1900004	Japan	SNP	425/191	$P = 0.028$	Associated with NTG
Chen et al. 2012 [88]		rs3858145	China	SNP	142/289	OR = 2.69,	Showed interaction with RFTN1 rs690037
						$P < 0.05$	
	10q21.3–22.1	rs61854782				B = −0.088,	Associated with VCDR in controls but not POAG
						$P = 0.004$	
Fan et al. 2011 [69]		rs1900004	US-Caucasian	SNP	539/336	OR = 1.89,	Associated with increased optic nerve area
						$P = 0.025$	
Dimasi et al. 2012 [71]		rs1900004	AU, NZ	SNP	873/886	OR = 1.12′	No association
						$P = 0.18$	
		rs3858145				OR = 1.13,	
						$P = 0.12$	
Wiggs et al. 2011 [59]	*CAV1/CAV2*	rs4236601	US-Caucasian	SNP	1000/1183	OR = 1.31,	Significantly associated in women than men
						$P = 0.0007$	
	7q31.1	rs1052990				OR = 1.25,	Significantly associated in women and nominally associated with NPG ($p = 0.039$)
						$P = 0.0084$	
Cao et al. 2012 [53]		rs4236601	African-Caribbean	SNP	272/165	OR = 1.15,	No association
						$P = 0.3332$	
Loomis et al. 2014 [61]		rs4236601	US-Caucasian	SNP	R1: 976/2132	$P_{meta} = 2.61 \times 10^{(-7)}$	Associated with early paracentral VF defect
		rs17588172			R2: 1140/2290	$P_{women} = 1.59 \times 10^{(-5)}$	
						$P_{meta} = 1.07 \times 10^{(-4)}$	Associated with early paracentral VF defect
Kuehn et al. 2011 [60]		rs4236601	US	SNP	545/297	$P = 0.5$	No association

(continued)

Table 22.1 (continued)

Studies	Gene/ chromosome	SNP ID	Population[a]	Study type	Study size (POAG/ controls)[a]	OR/Beta, *P* value	Any clinical association[a]
Cao et al. 2012 [53]	*CARD10*	rs9607469	African-Caribbean	SNP	272/165	OR = 1.13,	No association
	22q13.1					$P = 0.5096$	
Cao et al. 2012 [53]	*CDC7/TGFBR3*	rs1192415	African-Caribbean	SNP	272/165	OR = 1.14,	No association
						$P = 0.4802$	
Dimasi et al. 2012 [71]	*1p22*	rs1192415	AU, NZ	SNP	873/886	OR = 1.22,	Showed nominal significance with optic disc area
						$P = 0.03$	
Cao et al. 2012 [53]	*CDKN2B (−AS1)*	rs1063192	African-Caribbean	SNP	272/165	OR = 0.39,	Minor allele was protective against POAG
						$P = 0.0008$	
		rs4977756				OR = 0.89	No association
						$P = 0.4507$	
Fan et al. 2011 [69]	*9p21*	rs1063192	US-Caucasian	SNP	539/336	OR = 0.73,	Associated with decreased VCDR and POAG risk
						$P = 0.0006$	
Mabuchi et al. 2012 [70]		rs1063192	Japan	SNP	425/191	β = 0.11,	Associated with VCDR; and NTG ($p = 0.023$)
						$P = 0.0043$	
Dimasi et al. 2012 [71]		rs1063192	AU, NZ	SNP	873/886	OR = 0.74,	More strongly associated with advanced open-angle glaucoma
						$P = 2.2 \times 10^{-5}$	
Burdon et al. 2012 [72]		rs10120688rs7049105	AU, NZ	SNP	1432/595	VCDR –	Associated with larger VCDR and lower IOP
						β = 0.016,	
						$P = 0.03$;	
						IOP –	
						β = −2.135, $P = 0.001$	
Mabuchi et al. 2012 [70]	*CHEK2*	rs1547014	Japan	SNP	425/191	β = 0.11,	Associated with VCDR and HTG ($p = 0.013$)
						$P = 0.0079$	
Dimasi et al. 2012 [71]	*22q12.1*	rs1547014	AU, NZ	SNP	873/886	OR = 0.98,	No association
						$P = 0.77$	
Dimasi et al. 2012 [71]	*COL5A1/RXRA*	rs1536482	AU, NZ	SNP	873/886	OR = 0.94,	No association
						$P = 0.46$	
	9q34.2-q34.3	rs7044529				OR = 1.00,	
						$P = 0.98$	

Desronvil et al. 2010 [97]	*COL8A2* *1p34.2*	rs274754	US-Caucasian	SNP	100	$P = 0.018$	Associated with corneal thickness
Dimasi et al. 2010 [96]	*FBN1* *15q21.1*	rs17352842	AU-Caucasian	SNP	956	$P = 0.02$	Associated with CCT
Rocha et al. 2011 [102]	*GSTT1/GSTM1*	null > positive	Brazil	SNP	87/85	OR = 2.4, $P = 0.016$	T1 M0 genotype associated with higher IOP and severe defect of right eye optic nerve and visual field
Juronen et al. 2000 [103]	*1p13.3*	positive > null	Estonia	SNP	250/202	OR = 1.83, $P = 0.002$	GSTM1 were at significant risk for glaucoma and even higher in smokers (OR = 3.86, $P = 0.012$)
Jansson et al. 2003 [104]		positive = null	Sweden	SNP	200/200	P = ns	No association
Fan et al. 2010 [105]		positive = null	China	SNP	405/201	P = ns	No association
Liu et al. 2010 [106]	*NTF4*	Whole gene	US-Caucasian	SNP	443/533	–	Not associated with POAG
Rao et al. 2010 [107]		Whole gene	India	SNP	141/285	$P = 0.2$	No association
Vithana et al. 2010 [108]	*19q13.33*	c.338 T > C	China	SNP	174/91	–	Rare cause of POAG in Chinese
Chen et al. 2012 [94]		c.470G > C c.545C > T	China	SNP	720/230		May be a rare cause of POAG
Aung et al. 2002 [109]	*OPA1*	rs166850 rs10451941	UK	SNP	163/86	OR = 3.1, $P = 0.002$ $P = 0.03$	rs166850 combined with rs10451941 was more strongly associated with NTG ($p = 0.00001$)
Mabuchi et al. 2007 [110]	*3q28-q29*	rs10451941	Japan	SNP	285/185	OR = 2.27, $P = 0.004$	Increased risk of NTG; and age at diagnosis in HTG ($p = 0.048$)
Yao et al. 2006 [111]		rs166850 rs10451941	African-Caribbean	SNPs	109/48	P = ns	No association
Fan et al. 2010 [105]		rs166850 rs10451941	China	SNP	405/201	P = ns	No association
Dimasi et al. 2010 [96]	*PAX6* *11p13*	rs3026398	AU-Caucasian	SNP	956	$P = 0.02$	Associated with CCT; more strongly with rs662702 haplotype ($P = 0.009$)

(continued)

Table 22.1 (continued)

Studies	Gene/ chromosome	SNP ID	Population[a]	Study type	Study size (POAG/ controls)[a]	OR/Beta, *P* value	Any clinical association[a]
Chen et al. 2012 [95]	*PLXDC2*	rs7081455	China	SNP	462/577	OR = 1.25, *P* = 0.31	No association
Cao et al. 2012 [53]	*10p12.31*	rs7081455	African-Caribbean	SNP	272/165	OR = 1.04, *P* = 0.8052	No association
Chen et al. 2012 [88]	*RFTN1* *3p24.3*	rs3858145	China	SNP	142/289	ß = 25.66, *P* = 0.029	Associated with CCT
Fan et al. 2011 [69]	*SIX1/SIX6*	rs10483727	US-Caucasian	SNP	539/336	OR = 1.33, *P* = 0.0043	Associated with increased VCDR and POAG risk
Dimasi et al. 2012 [71]		rs10483727	AU, NZ	SNP	873/886	OR = 1.38, $P = 6.2 \times 10^{-6}$	Strongly associated with open-angle glaucoma
Cao et al. 2012 [53]	*14p22–23*	rs10483727	African-Caribbean	SNP	272/165	OR = 0.77, *P* = 0.4151	No association
Mabuchi et al. 2012 [70]		rs10483727	Japan	SNP	425/191	*P* = 0.017	Associated with age at diagnosis in NTG
Carnes et al. 2014 [79]		rs10483727	US-Caucasians	SNP	262/256	OR = 1.32, $P = 3.87 \times 10^{-11}$	Significantly associated with POAG
		rs33912345		SNP		OR = 1.27, $P = 4.2 \times 10^{-10}$	Associated with POAG; and thickness of retinal nerve fiber layer
Mabuchi et al. 2011 [51]	*SRBD1*	rs3213787	Japan	SNP	370/191	*P* = 0.0003 in NTG and *P* = 0.0013 in HTG	Associated with HTG and NTG including late-onset
Cao et al. 2012 [53]	*2p21*	rs3213787	African-Caribbean	SNP	272/165	OR = 0.45, *P* = 0.2882	None
Takano et al. 2012 [112]	*TLR4*	rs2149356	Japan	SNP	449/107	*P* = 0.000058	Associated with NTG
Chen et al. 2012 [95]		rs7037117	China	SNP	462/577	OR = 0.99, *P* = 0.99	No association
Shibuya et al. 2008 [113]	*9q33.1*	rs7037117	Japan	SNP	215/318	*P* = 0.0095	1.47- to 1.65-fold increased risk of NTG; strongest association with rs10759930 haplotype
Cao et al. 2012 [53]		rs7037117	African-Caribbean	SNP	272/165	OR = 0.73, *P* = 0.0571	No association

Sharma et al. 2012 [65]	*TMCO1*	rs4656461	AU, NZ	SNP	1420	ß = −2.56, $P = 0.004$	Correlation with age at diagnosis
Ozel et al. 2014 [64]	*1q24*	rs7518099	US-Caucasian	SNP	6.236	$P = 8 \times 10^{-8}$	Strongly associated with IOP
Chen et al. 2012 [95]	*TMTC2*	rs7961953	China	SNP	462/577	OR = 1.15, $P = 0.35$	No association
Cao et al. 2012 [53]	*12q21.31*	rs7961953	African-Caribbean	SNP	272/165	OR = 0.89, $P = 0.5559$	No association
Fan et al. 2010 [105]	*TNFα*	rs1800629	China	SNP	405/201	$P = 0.012$	Associated with HTG
Wang et al. 2012 [114]		rs4645836	China	SNP	234/230	OR = 0.63, $P = 0.017$	Protective for POAG
Mossböck et al. 2006 [115]	*6p21.3*	rs1800629	AU	SNP	114/228	OR = 0.96, $P > 0.05$	Not associated among Caucasian
		rs361525				OR = 0.52, $P > 0.05$	
Rao et al. 2010 [107]	*VAV2*	rs2156323	India	SNP	141/285	$P = 0.533$	No association
	9q34.1						
	VAV3	rs2801219				$P = 0.133$	
	1p13.3						
Dimasi et al. 2012 [71]	*ZNF469*	rs12447690	AU, NZ	SNP	873/886	OR = 1.01, $P = 0.91$	No association
	16q24	rs9938149				OR = 0.94, $P = 0.46$	
Chen et al. 2012 [95]	*ZP4*	rs693421	China	SNP	462/577	OR = 0.98, $P = 0.31$	No association
Cao et al. 2012 [53]	*1q43*	rs547984	African-Caribbean	SNP	272/165	OR = 1.05, $P = 0.7374$	No association
Kim et al. 2014 [98]		rs693421	Korea	SNP	211/904	OR = 1.4, $P = 0.0082$	Associated with POAG
Li et al. 2015 [58]	*CDKN2B-AS1*	rs2157719	Saudi Arabia	SNP	R[b]: 236/655	OR = 1.24, $P = 0.146$	–
	9p21						
	CDC7-TGFBR3	rs1192415				OR = 1.24, $P = 0.146$	–
	1p22						
	FNDC3B	rs4894796				OR = 1.03, $P = 0.779$	–
	3q25.31						

(continued)

Table 22.1 (continued)

Studies	Gene/ chromosome	SNP ID	Population[a]	Study type	Study size (POAG/ controls)[a]	OR/Beta, *P* value	Any clinical association[a]
Neamatzadeh et al. 2015 [116]	*TP53*	rs1042522	Iranian	SNP	65/65	OR = 2.1,	Pro72 allele is associated with POAG risk
	17p13.1					$P < 0.05$	
Emam et al. 2014 [117]	*NOS3*	rs2070744	Egypt	SNP	160/110	OR = 1.86,	rs2070744 is associated with high tension glaucoma; and with plasma nitrite/nitrate levels ($P < 0.001$)
						$P < 0.0001$	
	7q36	rs1799983				OR = 1.28,	
		27 bp-VNTR-a/b				$P = 0.21$	
						OR = 0.81,	
						$P = 0.33$	
Abu-Amero et al. 2013 [118]	*CAT*	rs1001179	Saudi Arabia	SNP	225/403	OR = 0.81,	Associated with age of onset, trend toward IOP, and duration of glaucoma
	11p13					$P = 0.218$	
Abu-Amero et al. 2014 [119]	*SOD2*	rs4880	Saudi Arabia	SNP	226/403	OR = 1.0,	Trend toward age of onset and IOP
	6q25.3					$P = 0.988$	
Abu-Amero et al. 2012 [62]	*CAV1/CAV2*	rs4236601	Saudi Arabia	SNP	220/405	OR = 1.06,	–
	7q31					$P = 0.699$	
Abu-Amero et al. 2012 [121]	*LOXL1*	rs1048661	Saudi Arabia	SNP	96/101	$P = 0.866$	–
	15q24.1	rs3825942				$P = 0.477$	
		rs2165241				$P = 0.176$	
Abu-Amero et al. 2006 [120]	*MYOC*	22,259 G/T (G324 V)	Saudi Arabia	SNP	27/96	$P = 0.74$	–
	1q24.3						
	OPTN	412 G/A (T34 T)				$P = 0.61$	–
	10p13	469 G/C (Q53H)				$P = 0.28$	–
Zanon-Moreno et al. 2013 [122]	*SLC23A2*	rs1279683	Mediterranean	SNP	250/250	OR = 2.47,	Associated with POAG risk and plasma vitamin C levels ($p < 0.001$)
	20p13					$P < 0.001$	
	TTPA	rs6994076				OR = 1.38,	Associated with plasma vitamin E levels ($p < 0.001$)
	8q12.3					$P = 0.122$	
	SEC14L2/TAP	rs737723				OR = 2.24,	Associated with POAG risk and nominal ($p = 0.047$) gene-gene interaction with SNP rs1279683
	22q12.2					$P < 0.001$	
	GPX4 19p13.3	rs757228				OR = 0.80,	–
						$P = 0.337$	

Zanon-Moreno et al. 2011 [123]	*RBP1*	rs176990	Mediterranean	SNP	150/150	OR = 0.97,	–
	3q23					*P* = 0.826	
		rs190910				OR = 0.83,	–
						P = 0.315	
	SLC23A1	rs10063949				OR = 1.19,	–
	5q31.2					*P* = 0.552	
	SLC23A2	rs1279683				OR = 1.67,	Associated with POAG risk and plasma vitamin C levels ($p < 0.001$)
	20p13					*P* = 0.010	
Abu-Amero et al. 2008 [124]	*GSTT1/GSTM1*	T0 M0	Saudi Arabia	SNP	49/120	OR = 5.67,	GSTT1 and GSTM1 positive genotypes are at risk for POAG
						P = 0.06	
		T1 M0				OR = 10.2,	
	1p13.3					*P* = 0.00001	
		T0 M1				OR = 11.3,	
						P = 0.00001	
Unal et al. 2007 [125]	*GSTT1/GSTM1*	T0 M1	Turkey	SNP	144/121	OR = 3.46,	GSTM1 positive and GSTT1 null genotypes are associated with increased risk of POAG
	1p13.3					*P* < 0.005	
Al-Dabbagh et al. [126]	*APOE*	rs429358	Saudi Arabia	SNP	60/130	OR = 2.75,	*APOE4* allele is a risk factor for POAG
	19q13.2	rs7412				*P* = 0.034	
Saglar et al. 2009 [127]	*APOE*	rs429358	Turkey	SNP	75/119	*P* = 0.38	–
	19q13.2	rs7412					
	TP53	rs1042522			73/90	*P* = 0.12	–
	17p						
Nilforoushan et al. [128]	*MTHFR 1p36.3*	rs1801133	Iran			*P* = 0.337	

[a]*AU* Australia, *CCT* central corneal thickness, *D* discovery cohort, *GE* Germany, *HPG* high-pressure glaucoma, *HTG* high-tension glaucoma, *IOP* intraocular pressure, *POAG* primary open-angle glaucoma, *NPG* normal-pressure glaucoma, *NTG* normal-tension glaucoma, *NL* Netherlands, *NZ* New Zealand, *R* replication cohort, *SG* Singapore, *SW* Sweden, *UK* United Kingdom, *US* United States, *VCDR* vertical cup-to-disc ratio

[b]Part of an International Glaucoma Genetics Consortium Replication Study

population using a candidate-gene approach [53, 69–72]. These SNPs are located in a noncoding gene, *CDKN2BAS*, near the tumor suppressor genes *CDKN2A/B*. The expression of CDKN2B could be induced by TGF-ß which is known to be involved in apoptosis in the developing retina and optic nerve and may thereby play an important role in glaucoma pathogenesis [73, 74]. Interestingly, carriers of the risk alleles for glaucoma are associated with larger VCDR [75, 76] and demonstrated lower IOP in comparison to non-carriers [72]. Based on these observations, it has been postulated that the *CDKN2B/CDKN2B-AS1* region of 9p21 may possibly predispose the RGC and ONH to glaucomatous optic neuropathy in a mechanism entirely independent of IOP and highlights the importance of chromosome 9p21 susceptibility locus as a risk factor for POAG [77].

Recently, Li et al. performed a GWAS on 3504 POAG cases and 9746 controls with replication of the most significant findings in 9173 POAG cases and 26,780 controls across 18 collections of Asian, African, and European descent including a replication cohort from our center in Saudi Arabia [58]. Apart from confirming strong evidence of association at *CDKN2B-AS1* (rs2157719, OR = 0.71, $P = 2.81 \times 10^{-33}$), the study also identified SNP rs1192415 in *CDC7-TGFBR3* gene (1p22) showing significant association to POAG (OR = 1.13, $P = 1.60 \times 10^{-8}$) in the Asian, African, and European population and SNP rs4894796 in *FNDC3B* (3q25.31) showing significant association in Asians only (OR = 0.89, $P = 7.93 \times 10^{-8}$). Interestingly, these results were found to be nonsignificant in the Saudi replication cohort indicating that the genetic cause for POAG in Saudi population may be different than those from Asian, African, and European descent.

GWAS studies by Wiggs et al. and Osman et al. in the Caucasian POAG and Japanese POAG cases, respectively, have demonstrated a strong association of SNP rs10483727 located in the intergenic region between the *SIX1* and *SIX6* locus (14q23) [56, 66]. SIX6 has been shown to express in the developing and adult human retina [78]. Moreover, the association of SNP rs10483727 in the *SIX1/SIX6* region has also been replicated in other Caucasian POAG cohort [69–71, 79] but not in the Afro-Caribbean subjects [53]. After the *CDKN2B-AS1* locus on chromosome 9, the *SIX1/SIX6* locus has since shown reproducible association with POAG and so it would be interesting to know if this locus is associated with POAG in the Saudi or other middle east population. However, currently there are no published reports of association of *SIX1/SIX6* locus with POAG in the middle east population.

Recently, *11p11.2* (containing multiple genes), *ABCA1*, *ABO*, *AFAP1*, *ARHGEF12*, *FAR2*, *GGA3*, *GMDS*, *PKDREJ*, and *PMM2* were added to the newly discovered genes associated with POAG [80–84]. These variants were significantly associated with glaucoma and the related functional visual field loss that could be future study targets for glaucoma patients in the middle east.

22.6 GWAS and Quantitative Endophenotype Traits

Genetic evaluation of quantitative endophenotype traits is often very useful in complex multifactorial diseases to understand the contribution of specific traits to the overall disease phenotype. Similar strategy has been successfully used in POAG to understand the contribution of proposed endophenotypes including IOP, VCDR, optic disc area, and CCT to the overall disease process. GWASs have been performed to examine the genetic components of these endophenotypes in POAG and normal population. van Koolwijk and colleagues performed a GWAS for IOP in POAG patients of European descent and identified SNPs rs11656696 and rs7555523, located in *GAS7* and *TMCO1*, respectively, suggesting a role for these two genes in IOP regulation [57]. Other loci found to be associated with IOP so far include *FNDC3B*, *ABCA1*, *ABO*, *11p11.2*, and *ARHGEF12* [82, 84]. Another three loci *FAR2*, *GGA3*, and *PKDREJ* did not reach a genome-

wide significance level ($P < 10^{-5}$) [80]. Three independent GWASs have evaluated the association of optic disc parameters (VCDR and optic disc area) in the normal general population. The loci associated included *ATOH7*, *CDC7/TGFBR3* and *SALL1*, *CARD10* for optic disc area, and *CDKN2B*, *SIX1*, *SCYL1/LTBP3*, *CHEK2*, and *DCLK1*, in addition to *ATOH7*, for VCDR [75, 76, 85, 86]. An exome sequencing also reported *SIX6* locus to influence VCDR ($P = 7.74 \times 10^{-7}$) [87]. A subsequent meta-analysis of the Rotterdam Study with the TwinUK study [76] demonstrated a strong association of *ATOH7*, *CDKN2B*, and *SIX1* in POAG with borderline association for *CDC7/TGFBR3* and *SALL4* (both $P = 0.04$). *CARD10* was not found to be associated with Afro-Caribbean POAG cases [53], whereas *CHEK2* was reported to be associated with VCDR and HTG among the Japanese [70] but not in Europeans [71]. Moreover, multiple studies have provided strong evidence of association of *ATOH7* [53, 69, 70, 88], *CDKN2B*(*−AS1*) [53, 69–72], and *SIX1/SIX6* [69, 71] with POAG. CCT is an important risk factor for POAG in individuals with increased IOP, and over 26 loci have been reported [89]. GWASs have identified several associated with CCT in the normal general population (Asian and European descent) and POAG cases (US Caucasians). These loci include *ZNF469*, *COL5A1*, *AKAP13*, *AVGR8*, and *COL8A2* [90–92]. The *ZNF469* and *COL5A1* have been found to be associated with CCT in both the Caucasian and Asian cohorts [90, 91].

The possible role of these newly discovered loci associated with POAG and its endophenotypes in understanding the pathophysiology of POAG has been elegantly reviewed by Iglesias et al. elsewhere [93]. The review integrates current knowledge in POAG from human and experimental data and dissects the known molecular pathways and biological processes pointed by genetic association studies including (1) extracellular matrix metabolism (ECM), (2) transforming growth factor-β (TGF-β) signaling, (3) tumor necrosis factor-α (TNF-α) signaling, and (4) estrogen metabolism and vascular tone pathway and describes how the newly discovered genes contribute to these and other pathways.

22.7 Candidate Genes and POAG

Recent reviews by Takamota and Araie [31] and Janssen et al. [30] presented a list of genes identified from numerous GWAS and association studies thus far. Taken together, the list of almost 50 genes may represent highly likely candidate genes that may be involved in POAG pathogenesis. Many studies have been performed to replicate the GWAS findings in the Asian, Afro-Caribbean, and Caucasian/European population using the candidate gene approach [51, 53, 59–61, 64, 65, 69–72, 94–97]. And also, many studies were performed to test the association of specific known gene/SNP with POAG using the same approach in different populations including the middle east [53, 62, 94–96, 98–128]. These SNP replication and genetic association studies in the middle east and other populations are also listed in Table 22.1. From among them consistent findings have been reported for *ATOH7* [53, 69, 70, 88], *CDKN2B*(*−AS1*) [53, 69–72], *GSTT1/GSTM1* [102, 103, 124, 125], *SIX1/SIX6* [69, 71], and *TMCO1* [64, 65] loci indicating a potential role of these genes/loci in the pathogenesis of POAG. However, except for the glutathione-S transferase (GST) polymorphism, none of these loci have been either found to be associated with POAG (e.g., *CAV1/CAV2*, *CDC7/TGFBR3*, *FNDC3*) or has not been reported yet (e.g., *ATOH7*, *CDKN2B*(*−AS1*), *SIX1/SIX6*, *TMCO1*) in the middle east population. However, the positive findings of *GSTT1* and *GSTM1* genotypes in the middle east population may be very interesting, highlighting the role of antioxidants and/or oxidative stress-related pathways/mechanisms in the pathogenesis of POAG in this population. This view is strongly supported by recent meta-analysis studies that examined the association of GST polymorphisms and risk of POAG [129–131]. We have previously studied SNPs in two of the antioxidant genes, *CAT* (rs1001179) and *SOD2* (rs4880) [118, 119], in the Saudi POAG

patients. Although the studies did not provide any direct association with POAG but indicated a trend toward an association with IOP and age of onset of POAG. In addition, some studies have demonstrated moderate evidence for association of SNPs in *TP53*, *NOS3*, *SEC14L2/TAP*, and *APOE* [116, 117, 122, 126]. However, these studies have been limited by sample size and would need further investigations in a large population-based cohort. Examination of causative gene such as *MYOC*, *OPTN*, and *LOXL1* in Saudi POAG cases has also provided negative results [120, 124].

22.8 Final Remarks

There is significant progress in understanding the genetic basis of POAG, largely due to the application of GWAS methodology in different population. In recent years, GWAS have identified several loci associated with POAG including *CAV1/CAV2*, *TMCO1*, *CDKN2B-AS1*, *CDC7-TGFBR3*, *SIX1/SIX6*, *GAS7*, and *ATOH7*.

Associations between the *CDKN2B*(*−AS1*) locus on chromosome 9p21 and POAG has been extensively established across different population and represents a major genetic risk factor for POAG. Studies involving the *SIX1/SIX6* and the *ATOH7* loci affecting the optic disc parameters and POAG itself have also been reproducible. Other loci seem to be more of ethnic specific. *CAV1/CAV2* and *CDC7-TGFBR3* loci do not seem to contribute to POAG in the middle east, and the role of other newly discovered loci is yet to be established. Moreover, the *GSTT1/GSTM1* genotypes were found to be strongly associated with POAG in the middle east population and may be more studies are needed to examine the role of oxidative stress and antioxidant pathways in this population.

Based on the current and new genes identified in glaucoma, it may be possible to develop an algorithm of SNP risk scores to assess the future risk of POAG in patients that could be clinically useful. However, despite the tremendous progress, the genetic basis of POAG is still not completely understood, and further investigations are needed to identify novel genes and pathways contributing to glaucoma that may help define disease-specific targets and facilitate the development of diagnostic and therapeutic strategies.

Declaration of Interest The authors report no conflict of interest.

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Corneal Dystrophies in India

23

Namrata Sharma and Arundhati Sharma

Abstract

Corneal dystrophies are a heterogeneous group of hereditary disorders resulting in loss of vision due to slow progressive corneal opacification. New insights into the molecular basis changed the perception and led to a new International Committee for Classification of Corneal Dystrophies (IC3D) based on phenotypic description, pathologic characteristics and genetic knowledge. Several studies indicate existence of genomic diversity in India and therefore existence of a different mutation spectrum was expected for the dystrophies. The current chapter describes mutations in the TGFBI, CHST6, SLC4A11, COL8A2, ZEB1 and TCF4 genes causing corneal dystrophy in the patients and compares the mutational spectrum between north and south India.

Keywords

Corneal dystrophy • Genetics • Mutational analysis • Dominant • Recessive • Heterogeneity

Corneal dystrophies (CDs), a heterogeneous group of hereditary disorders that result from the loss of corneal transparency, are generally bilateral and noninflammatory with slow progressive corneal opacification. Traditionally, they are categorized on the basis of anatomy of the corneal layers affected and manifest as autosomal dominant, recessive, or X-linked recessive with variable penetrance and phenotype diversity. Discovery of a molecular basis changed the perception of CDs and led to a new International Committee for Classification of Corneal Dystrophies (IC3D) [1] which integrates phenotypic description, pathologic characteristics, and genetic knowledge of the dystrophies.

23.1 Indian Perspective

Several studies have indicated existence of genomic diversity in India, and various analyses of population structure documented that India is an

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ethnically and linguistically diverse country, and the North and South Indian populations are genetically distinguishable [2, 3]. Therefore, existence of a different mutation spectrum is expected for various disorders which differentiate the two populations. Extensive studies on the corneal dystrophies have been carried out in India, and the major regions covered are shown in Fig. 23.1.

23.1.1 The Autosomal Dominant Corneal Dystrophies

The autosomal dominant dystrophies are caused by mutations in the *TGFBI* (OMIM: 601692) gene which codes for an extracellular "keratoepithelin," a 683 amino acid, 69-kDa matrix protein expressed in many tissues including the corneal epithelium. The protein has an

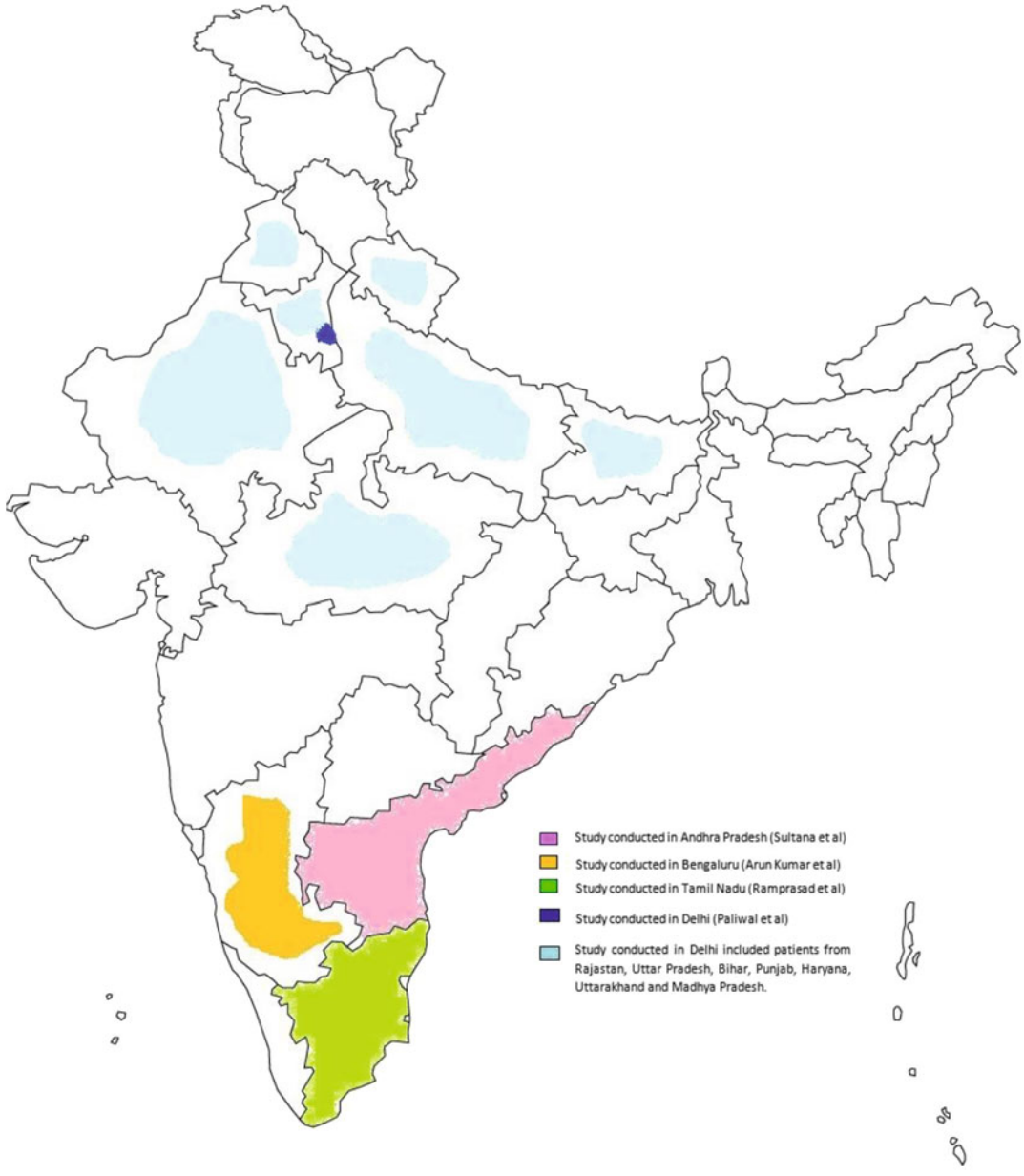

Fig. 23.1 Shows the north and south regions of India screened for the corneal dystrophies

NH_2-terminal signal peptide, four fasciclin-like domains, and a COOH-terminal Arg-Gly-Asp sequence which is a putative integrin-binding motif. Majority of the *TGFBI* mutations are located in the Fas1 domain 4 code for abnormal keratoepithelin protein, accumulation of which leads to the diseased state [4].

23.2 Mutation Screening of the *TGFBI* Gene

A study from South India, on 37 patients of whom 18 were lattice corneal dystrophy (LCD) and 19 granular corneal dystrophy (GCD), revealed mutations in 14 of 18 LCD and all GCD patients [5].

In LCD, three novel heterozygous mutations Gly594Val, Val539Asp, and Val624-Val625del were identified in patients with atypical clinical features. Other mutations documented were Arg124Cys and His626Arg. Eighteen patients with GCD had Arg555Trp mutation GCD type III (Reis-Bucklers dystrophy) and had the Arg124Leu mutation. Seven novel SNPs were also found, of which a change of Leu269Phe was seen in 12 of 18 patients with the Arg555Trp mutation.

In another study on nine individuals from two South Indian families with GCD, mutation analysis of exon 12 *TGFBI* identified Arg555Trp mutation in all affected members, of which five patients were homozygous [6]. Clinical examination revealed a severe form of GCD with early-onset and superficial lesions in the homozygous individuals and a milder phenotype in the heterozygous individuals. Histopathologic evaluation of corneal specimens from homozygous patients confirmed the presence of superficial granular deposits. Genotype-phenotype correlation and comparison with earlier reports highlight uniform expression of the Arg555Trp allele in homozygous individuals with a severe phenotype which can also be recognized based on clinical and histopathologic features.

In another study, a case of ACD was diagnosed on clinical, histopathological, and microscopic observations, but *TGFBI* screening did not reveal any mutation [7].

A study from North India on 80 patients with granular and/or lattice corneal dystrophies [8] documented 57 granular CD or its variants and 23 LCDs.

Arg555Trp and Arg124Cys were the most common mutations documented accounting for 44% of GCD and 24% of LCD, respectively, along with mutations Thr538Pro and Arg124His for the first time from India (PP). Other changes included Arg124Leu, His626Arg, and Ala546Asp (Table 23.1) and two single nucleotide polymorphisms (SNPs) rs4669 and rs11331170. Two novel mutations Ser516Arg and Leu559Val were identified in GCD and in a case with suspected GCD/epithelial basement membrane dystrophy, respectively. Mutations were not identified in eight patients of whom six were Bowman layer dystrophy and two lattice dystrophy.

A novel *TGFBI* variant with Arg124Leu mutation identified in a family diagnosed with Bowman layer dystrophy showed diffuse anterior stromal haze and superficial reticular opacities of both hyaline and amyloid deposits. Avellino corneal dystrophy (ACD) with Arg124His mutation was also reported from two North Indian families.

Ser516Arg mutation was associated with multiple, small discrete opacities in the anterior part with clear intervening stroma and limbus and no photophobia or corneal erosions. The Ser516Arg change affects the first base of exon 12 and may influence pre-mRNA splicing, leading to exon skipping or intron retention, thereby resulting in altered protein structure that could possibly explain the different phenotype of the patient from North India.

A highly variable phenotype not conforming to typical GCD or its subtypes was seen in the Leu559Val mutation with a map pattern fingerprint-like structure in the subepithelial region along with granular gray-white opacities in the inferonasal quadrant overlying the papillary areas with a beaten metal appearance of the endothelium. Reports of mutation at the adjacent

Table 23.1 *TGFBI* mutations in the Indian population

S. No.	Nucleotide change	Amino acid change	Author (year)
1.	c.1706T>G	Leu569Arg	Warren et al. (2003)
2.	c.1616T>A	Val539Asp	Chakravarthi et al. (2005)
3.	c.1781G>T	Gly594Val	Chakravarthi et al. (2005)
4.	c.1870_1875del GTGGTC	Val624_Val625del	Chakravarthi et al. (2005)
5.	c.1924 A>G	His626Arg	Chakravarthi et al. (2005) and Paliwal et al. (2010)
6.	c.417 C>T	Arg124Cys	Chakravarthi et al. (2005)
7.	c.1710 C>T	Arg555Trp	Chakravarthi et al. (2005)
8.	c.852 C>T	Leu269Phe	Chakravarthi et al. 2005
9.	c.418 G>T	Arg124Leu	Chakravarthi et al. (2005) and Paliwal et al. (2010)
10.	c.1548C>G	Ser516Arg	Paliwal et al. (2010)
11.	c.1675T>G	Leu559Val	Paliwal et al. (2010)
12.	c.1637C>A	Ala546Asp	Paliwal et al. (2010)
13.	c.1612A>C	Thr538Pro	Paliwal et al. (2010)
14.	c.367G> C	Arg124His	Paliwal et al. (2010)
15.	c.356G>A	Cys119Tyr	Paliwal et al. (2010)
16.	c.1416 C>T	Leu472Leu	Paliwal et al. (2010)
17.	c.1667T>C	Phe540Phe	Paliwal et al. (2010)

codon (Leu558Pro) were shown to cause an atypical amyloidogenic phenotype [9].

Codons Arg124 and Arg555 of the *TGFBI* FAS1 domain are mutational hot spots [10], but these mutations do not substantially alter the protein structure and are likely to affect direct protein-protein interactions, whereas rare mutations may cause protein misfolding in the cell. Findings of corneal amyloid deposits in cases with LCD, but not in their skin, suggested that mutated KE interacts with distinctive factors in the cornea to form amyloid deposits characteristic of these dystrophies [11].

Difficulty arises in classification of cases with phenotypic variability, especially where histopathology and mutation data is not available. Such cases support the existence of genetic heterogeneity in the disease.

23.2.1 The Autosomal Recessive Corneal Dystrophies

23.2.1.1 Macular Corneal Dystrophy

MCD is an autosomal recessive disorder characterized by the presence of superficial, central, elevated, irregular whitish opacities (macules) giving the condition its name. Onset is usually in the first decade, starting with a fine superficial haze leading gradually to opacification in the central stroma which extends to the periphery involving the entire cornea and leading to visual impairment. In advanced stages, the corneal endothelium is affected and Descemet's membrane develops guttate excrescences. The stroma thickens from the imbibition of water due to endothelial decompensation leading to progressive loss of vision.

MCD, though identified throughout the world, is rare in most of the populations. Histopathology of MCD is typical and shows intracellular storage of glycosaminoglycans (GAGs) within keratocytes and the corneal endothelial cells. The extracellular deposition of similar material is seen in the corneal stroma and the Descemet's membrane. Ultrastructural features of MCD include the presence of randomly distributed lacunae in the affected corneas filled with clusters of abnormal sulfated chondroitinase. Immunochemical studies recognize three immunophenotypes, MCD type I, MCD type IA, and MCD type II, that are clinically and histopathologically indistinguishable from each other.

Mutations in carbohydrate sulfotransferase gene (*CHST6*; OMIM:605294) encoding an enzyme corneal N-acetylglucosamine-6-sulfotransferase (c-GlnNAc6ST) were identified which transfers a sulfate group to an unsulfated

KS proteoglycan [12]. Characteristic deposits of MCD are seen when this sulfation reaction fails and unsulfated proteoglycan precursors get accumulated in the cornea.

Although fairly established that *CHST6* mutations are responsible for MCD, all cases cannot be explained by coding region, splice site, or 5′ upstream region changes, and marked genetic heterogeneity in *CHST6* is documented in different populations of the world [13–16].

Molecular basis for the different immunophenotypes is also not elucidated, and studies on individuals affected with MCD types I, IA, and II found no relationship between immunophenotypes and specific *CHST6* mutations [17, 18].

23.3 Mutation Screening of the *CHST6* Gene

In a first study from South India, 75 MCD patients from 51 families were classified into MCD type I (70 patients of 47 families) and MCD type II (five patients of four families) based on serum AgKS levels [13]. Mutation analysis of *CHST6* coding region identified 20 different mutations (Table 23.2) which included homozygous missense, frameshift, homozygous replacement, deletion, and also compound heterozygous mutations. Mutations were not identified in four patients with MCD type II.

Another study from South India reported *CHST6* gene mutations in 36 patients from 31 families [19]. Consanguinity was seen in 20 families, and 14 were sporadic cases with 22 mutations identified which included deletions, insertions, and nonsense and missense mutations of which 17 were novel and two were compound heterozygous. Another study on 31 patients from 26 families identified 14 novel mutations (Table 23.2).

In contrast, a study from North India delete on 30 MCD families, reported consanguinity in 8 and a positive family history in 6, while the remaining 24 cases were sporadic [20].

CHST6 screening revealed 7 homozygous and 5 heterozygous nucleotide changes. Mutations were seen in 17 patients belonging to 11 families. Four novel homozygous missense mutations were identified in 9 individuals from 5 families. A heterozygous stop codon and compound heterozygous mutations were the other changes identified. Clinical features of the patients were similar to the features seen in individuals with homozygous *CHST6* mutations. The parents were carriers for either of the two changes. Reported mutations were identified in 5 individuals from 3 families, and a change c.320 T>C (Phe107Ser) resulting in a non-synonymous missense variant was identified in 11 patients and 21 controls, and changes were not seen in two individuals.

Numerous novel structural changes distributed along the length of *CHST6* coding region are documented till date, and a large proportion of these are reported from India. *CHST6* gene does not have any mutation hot spots, but several of the mutations lead to frameshift resulting in altered protein structure.

23.4 The Endothelial Dystrophies

The endothelium, a monolayer of specialized neural crest-derived cells critical to the development of anterior segment, keeps the stroma in a state of relative dehydration by actively pumping out water into the aqueous humor, thereby maintaining transparency of the cornea [21].

The endothelial corneal dystrophies result from primary endothelial dysfunction and include Fuchs' endothelial corneal dystrophy (FECD1, MIM136800; FECD2, MIM610158), posterior polymorphous corneal dystrophy (PPCD1, MIM122000; PPCD2, MIM609140; PPCD3, MIM609141), and congenital hereditary endothelial dystrophy (CHED: CHED1, MIM121700, and CHED2, MIM217700).

Congenital hereditary endothelial dystrophy (CHED) is a rare heritable disorder of the corneal endothelium characterized by bilateral, symmetrical, noninflammatory corneal clouding which ranges from diffuse haze to characteristic ground glass appearance, evident at birth or in subsequent years [1]. Degeneration of the

Table 23.2 *CHST6* mutations in the Indian population

S. No	Nucleotide change	Amino acid change	Author (year)
1.	c.708-732del	FS at R5	*Sultana* et al. (*2003*)
2.	c.744C>T	Gln18X	*-do-*
3.	c.786-792del	FS at P31	*-do-*
4.	c.847G>A	Gly52Asp	*-do-*
5.	c.850C>T	Ser53Leu	*Sultana* et al. *(2003) and Warren* et al. *(2003)*
6.	c.872delC	FS at F 60	*-do-*
7.	c.890delC	FS at V66	*-do-*
8.	c.985C>G	Ser98Trp	*-do-*
9.	c.986C>G	Phe107Ser	*-do-*
10.	c.1012 T>C	Phe107Ser	*-do-*
11.	c.1055C>G	Phe121 Leu	*-do-*
12.	c.1061G>A	Trp123X	*-do-*
13.	c.1151C>A	Cys153X	*-do-*
14.	c.1279 in. ACG	R195–196 ins	*-do-*
15.	c.1296C>A	Arg202Ser	*-do-*
16.	c.1303C>A	Pro204 Gln	*-do-*
17.	c.1304-1306del3ins AT	FS atP204	*-do-*
18.	c.1321C>T	Ser210Phe	*-do-*
19.	c.1353G>T	Asp221Tyr	*-do-*
20.	c.1348insCTG	Trp219-220ins	*-do-*
21.	c.1355C>G	Asp221Glu	*-do-*
22.	c.1617G>T	Gly309X	*-do-*
23.	c.1731G>T	Glu347X	*-do-*
24.	c.15_16delCG	Arg5fs	*Warren* et al. *(2003)*
25.	757T>G	Leu22Arg	*-do-*
26.	816C>T	His42Tyr	*-do-*
27.	840C>T	Arg50Cys	*-do-*
28.	841G>T	Arg50Leu	*-do-*
29.	c.198delC	Phe67fs	*-do-*
30.	970G>A	Arg93His	*-do-*
31.	982G>C	Arg97Pro	*-do-*
32.	997G>A	Cys102Tyr	*-do-*
33.	1071C>T	Arg127Cys	*-do-*
34.	1306G>A	Arg205Gln	*-do-*
35.	1308G>A	Ala206Thr	*-do-*
36.	1438A>C	His249Pro	*-do-*
37.	1512G>A	Glu274Lys	*-do-*
38.	Replacement	194 Asn Leu Arg 196;	*-do-*
	ACCTAC 1273GGT	194 Arg Cys 195	
39.	Replacement	Major	*-do-*
	GCG 1304AT		
40.	Deletion CG707/708	Major	*-do-*
41.	Deletion C890	Major	*-do-*
42.	Deletion A1237	Major	*-do-*
43.	Deletion1748–1770	Major	*-do-*
44.	Deletion of open reading frame	Absent	*-do-*
45.	c.6G>A	Trp2X	*Sultana* et al. *(2005)*
46.	c.7C>A	Leu3Met	*-do-*
47.	c.293C>T	Ser98Leu	*-do-*

(continued)

Table 23.2 (continued)

S. No	Nucleotide change	Amino acid change	Author (year)
48.	c.166G>A	Val56Arg	*-do-*
49.	c.167T>G	Val56Arg	*-do-*
50.	c.16_40del	Val6fs	*-do-*
51.	c.161C>T	Ser54Phe	*-do-*
52.	c.180delC	Phe60fs	*-do-*
53.	c.217G>A	Ala73Thr	*-do-*
54.	c.320T>C	Phe107Ser	*-do-*
55.	c.369G>A	Trp123X	*Sultana* et al. *(2005) and Paliwal* et al. *(2010)*
56.	c.391T>C	Ser131Pro	*-do-*
57.	c.459C>A	Cys153X	*-do-*
58.	c.494G>C	Cys165Ser	*-do-*
59.	c.495C>G	Cys165Trp	*-do-*
60.	c.495C>T	Cys153X	*-do-*
61.	c.500C>T	Ser167Phe	*-do-*
62.	c.533T>G	Phe178Cys	*-do-*
63.	c.545delA	Gln182fs	*-do-*
64.	c.578T>C	Leu193Pro	*-do-*
65.	c.604C>A	Arg202Ser	*-do-*
66.	c.611C>A	Pro204Gln	*-do-*
67.	c.611C>G	Pro204Arg	*-do-*
68.	c.656_657insCTG	Ala219_Arg220insTrp	*-do-*
69.	c.661G>T	Asp221Tyr	*-do-*
70.	c.663C>G	Asp221Glu	*-do-*
71.	c.814C>A	Arg272Ser	*-do-*
72.	c.827T>C	Leu276Pro	*-do-*
73.	c.1000C>T	Arg334Cys	*-do-*
74.	c.1002_1012delinsTTG	His335fs	*-do-*
75.	c.294 C>G	Ser98Leu	*-do-*
76.	c.161C>A	Ser54Tyr	*Paliwal* et al. *(2010)*
77.	c.173A>G	Gln58Arg	*-do-*
78.	c.176T>A	Leu59His	*-do-*
79.	c.877C>T	Leu293Phe	*-do-*
80.	c.278G>A	Arg93His	*-do-*
81.	c.820G>C	Glu274Gln	*-do-*
82.	c.290G>C	Arg97Pro	*-do-*
83.	c.65T>G+	Leu22Arg	*-do-*
84.	c.172C>T	Gln58X	*-do-*

corneal endothelium and the posterior non-banded part leads to corneal edema, especially of the stroma resulting in profound thickness of the Descemet's membrane. CHED occurs as an autosomal dominant (CHED1) (OMIM 121700) or a common but severe autosomal recessive (CHED2) (OMIM 217700) form. This distinction no longer holds true due to insufficient data on the presence of autosomal dominant form and the ICD3 now recognises the existence of only one form of CHED (OMIM 217700).

Mutations in the solute carrier family 4 (sodium borate cotransporter) member 11 (*SLC4A11*, OMIM 610206) cause CHED and corneal dystrophy and perceptive deafness (CDPD; OMIM 217400), i.e., CHED with hearing loss. The gene has 19 exons that encode the bicarbonate transporter-related protein 1 (BTR1) of 891 amino acids and 14 transmembrane domains with intracellular amino and carboxyl terminals. Functions attributed to BTR1 are maintenance of boron homeostasis, cell growth, and proliferation

and activation of the mitogen-activated protein kinase pathway [22].

23.5 Mutation Screening of the *SLC4A11*, *COL8A2*, *ZEB1*, and *TCF4 Genes*

There are several reports from South India on the role of *SLC4A11* in CHED causation: in a study on 49 affected individuals from 42 families, 27 mutations were identified in 35 families of which 19 were novel (Table 23.3). Seven families did not show any changes. Another study from the same region on 16 families identified mutations in 12, while four families did not show any changes [23, 24]. A study from the Chennai state of South India on 26 patients from 20 families identified ten mutations in 11 families of which three were novel. No mutations were identified in nine families. Similar study on five families from the same region identified mutations in all [25, 26]. Analysis of CHED in six families from the adjoining state of Karnataka revealed mutations in all the families of which six were novel mutations [27, 28]. On the contrary, the study from North India on 25 affected individuals from 20 families reported consanguinity in nine of the 20 families. Four mutations were identified in 14 patients from nine families of which one was novel. No variations either in the coding or in the upstream putative promoter region were identified in 11 patients from 11 families. The study also reported inter- and intrafamilial variability along with the presence of genetic heterogeneity [29]. None of the patients in the various studies had associated sensorineural hearing loss.

The most interesting feature of interfamilial variability in CHED seen in a family was the presence of variable phenotypes in the affected siblings homozygous for the splice site mutation c.2240+1G>A and a completely different phenotypic presentation in the heterozygous carrier mother. The mutation was reported as the single heterozygous change leading to CHED in an individual from the United Kingdom as the change was not identified in 50 British and 30 South Indian controls [30]. A plausible explanation for corneal opacities in some heterozygous carriers is that the mutation is a dominant change with variable expressivity and penetrance, or a rare polymorphism segregating with the disease.

Two other mutations c.2470G>A and c.1156T>C showed interfamilial and/or intrafamilial variability with no genotype-phenotype correlation. Similar reports from South India substantiate on the absence of evident correlations between clinical, histopathology, and *SLC4A11* mutations [19, 27] indicating that the phenotype may depend on the mutations, environmental factors, and other genes.

Heterozygous *SLC4A11* changes are documented in late-onset FECD in which haploinsufficiency and accumulation of aberrantly folded protein are reported [27]. PPCD also results from primary endothelial dysfunction, and a few cases are reported to present with corneal haze at birth [31] resulting in phenotypic overlap between CHED and PPCD [32]. Incomplete penetrance and *de novo* zinc finger E-box-binding homeobox 1 (*ZEB*1; OMIM 189909) mutations have been observed in PPCD.

FECD, the most prevalent of endothelial dystrophies, is characterized by bilateral primary corneal guttata, reduced endothelial cell density stromal edema, and progressive degeneration of the endothelium leading to a thickened Descemet's membrane with posterior excrescences known as corneal guttata. FECD has complex etiology, with involvement of genetic and environmental factors, familial clustering in ~50% cases, and autosomal dominant inheritance [32]. Onset is typically in the fifth decade, but a phenotypically different early-onset variant also exists [33].

Studies suggest that CHED, FECD, and PPCD are allelic variants of the same disease continuum and genetic interaction between genes modulate expressivity of the phenotype [34].

COL8A2 (OMIM: 120,252), a major component of Descemet's membrane, is implicated in early- and late-onset FECD [35]. *COL8A2*

Table 23.3 *SLC4A11* mutations in the Indian population

S. No.	Exon/intron (IVS)	Nucleotide change	Amino acid change	Author (year)
1.	4	c.353_356delAGAA	Lys118ThrfsX12	Vithana et al. (2006) and Arun kumar et al. (2007)
2.	IVS 15	c.2067 -6_-16 delins	Inactivation of splice	Vithana et al. (2006) and Hemadevi et al. (2008)
		GGCCGGCCGG	acceptor site	
			IVS15 –6_-16delins	
3.	8	c.859-862delGAGAinsCCT	E287fsX21	Arun kumar et al. (2007)
4.	11	1391G>A	Gly464Asp	Arun kumar et al. (2007)
5.	15	IVS15-6_-16delinsGGCCGGCCGG		Arun kumar et al. (2007)
6.	15	c.2014_2016delTTC orc.2017_2019delTTC	F672del or F673del	Arun kumar et al. (2007)
7.	17	g.9191G>A	Arg804His	Arun kumar et al. 2007
8.	17	g.9200delTinsGG	L807RfsX71	Arun kumar et al. (2007)
9.	2	c.246_247delTTinsA	Phe84LeufsX32	Jiao et al. (2007) and Arun kumar et al. (2007)
10.	13	c.1704_1705delCT	Ser569ArgfsX177	Jiao et al. (2007)
11.	14	c.1813C>T	Arg605X	Jiao et al. (2007) and Arun kumar et al. (2007)
12.	17	c.2264G>A	Arg755Gln	Jiao et al. (2007)
13.	18	c.2411G>A	Arg804His	Jiao et al. (2007
14.	18	c.2420delTinsGG	Leu807ArgfsX71	Jiao et al. (2007
15.	18	c.2606G>A	Arg869His	Jiao et al. (2007), Kodaganur et al. (2013) and Arun kumar et al. (2007)
16.	18	c.2498C>T	Thr833Met	Jiao et al. (2007) and Arun kumar et al. (2007)
17.	4	c.427G>A	Glu143Lys	Ramprasad et al. (2007)
18.	9	c.1156T>C	Cys386Arg	Ramprasad et al. (2007)
19.	17	c.2263C>T	Arg755Trp	Ramprasad et al. (2007)
20.	6	c.720G>A	Trp240X	Ramprasad et al. (2007)
21.	Intron 16	c. [2240+1G>A] + [?]	Inactivation of splice donor site	Ramprasad et al. (2007)
22.	Exon 17 and intron 17, resp.	c.[2398C>T] + [2437-1G>A]	p. Gln800X and inactivation of splice acceptor site	Ramprasad et al. (2007)
23.	2	c.140delA	Tyr47SerfsX69	Sultana et al. (2007)
24.	3	c.306delC	Gly103ValfsX13	Sultana et al. (2007)
25.	3	c.334C>T	Arg112X	Sultana et al. (2007)
26.	4	c.473_480del8 bp	Arg158ProfsX4	Sultana et al. (2007) and Hemadevi et al. (2008)
27.	5	c.618_619delAG	Val208AlafsX38	Sultana et al. (2007)
28.	5	c.625C>T	Arg209Trp	Sultana et al. (2007)
29.	5	c.638C>T	Ser213Leu	Sultana et al. (2007)
30.	6	c.697C>T	Arg233Cys	Sultana et al. (2007)
31.	7	c.878_889del12	Glu293_Glu296del	Sultana et al. (2007)
32.	IVS-7	c.996+26C_+44Cdel19	–	Sultana et al. (2007)
33.	IVS-8	c.1091–1G>C		Sultana et al. (2007)
34.	9	c.1202C>A	Thr401Leu	Sultana et al. (2007)

(continued)

Table 23.3 (continued)

S. No.	Exon/intron (IVS)	Nucleotide change	Amino acid change	Author (year)
35.	10	c.1253G>A	Gly418Asp	Sultana et al. (2007)
36.	10	c.1317_1322del6ins8	Leu440ValfsX6	Sultana et al. (2007)
37.		c.1418T>G	Leu473Arg	Sultana et al. 2007
38.	12	c.1466C>T	Ser489Leu	Sultana et al. (2007) and Arun kumar et al. (2007)
39.	13	c.1751C>A	His568HisfsX177	Sultana et al. (2007)
40.	14	c.1813C>T	Arg605X	Jiao et al. (2007)
41.	14	c.1894G>T	Glu632X	Sultana et al. (2007) and Arun kumar et al. (2007)
42.	17	c.2263C>T	Arg755Trp	Sultana et al. (2007), Hemadevi et al. (2008), and Ramprasad et al. (2007)
43.	17	c.2264G>A	Arg755Gln	Jiao et al. (2007), Ramprasad et al. (2007) and Arun kumar et al. (2007)
44.	17	c.2318C>T	Pro773Leu	Sultana et al. (2007) and Hemadevi et al. (2008)
45.	17	c.2389_2391delGAT	Asp797del	Sultana et al. (2007)
46.	17	c.2407C>T	Gln803X	Sultana et al. (2007)
47.	18	c.2470G>A	Val824Met	Sultana et al. (2007)
48.	18	c.2605C>T	Arg869Cys	Sultana et al. (2007) and Arun kumar et al. (2007)
49.	17	c.2623C>T	Arg875X	Sultana et al. (2007)
50.	4	c.374G>A	Arg125His	Hemadevi et al. (2008)
51.	4	c.478G>A	Ala160Thr	Hemadevi et al. (2008)
52.	5–6	c.654(−97)_c.778(−1488) del698nucleotides	C218KfsX49	Hemadevi et al. (2008)
53.	7	c.806C>T	Ala269Val	Hemadevi et al. (2008)
54.	9	c.1156T>C	Cys386Arg	Hemadevi et al. (2008)
55.	18	c.2506C>T	Gln836X	Hemadevi et al. (2008)
56.	19	c.2618T>C	Leu873Pro	Hemadevi et al. (2008)
57.	9	c.1156T>C	Cys386Arg	Paliwal et al. (2010)
58.	16 (splice site)	c.2240+1G> A	Inactivation of splice site	Paliwal et al. (2010)
59.	16	c.2470G>A	Val824Met	Paliwal et al. (2010)
60.	18	c.2470G>A	Val824Met	Paliwal et al. (2010)
61.	18	c2518-c2520 delCTG	Leu840del	Paliwal et al. (2010)
62.	6	c.785C>T	Thr262Ile	Kodaganur et al. (2013)
63.	10	c.1249G>A	Gly417Arg	Kodaganur et al. (2013)
64.	14	c.1831T>C	Cys611Arg	Kodaganur et al. (2013)
65.	15	c.2170C>G	His724Asp	Kodaganur et al. (2013)

mutations in these forms were not identified in different studies nor in two studies from India [36, 37, 38, 39].

Analysis of 82 FECD patients from North India for *ZEB1* and *TCF4* (OMIM 602272) variant revealed novel *ZEB1* change IVS2+276 C/T, a nonsense p. Leu947stop mutation, missense mutations p. Glu733Lys, p. Ala818Val, synonymous variation p. Ser234Ser, reported mutation p. Gln840Pro, and five polymorphisms. *TCF4* SNP rs613872 overrepresented in FECD patients was associated with endothelial cell density

showing a plausible role in FECD pathogenesis [39]. Another study on the Indian population shows significant association of *TCF4* SNP rs17089887 and (CTG) 18.1 intronic repeat expansions [40].

The role of *ZEB1* in FECD pathogenesis is not defined. *ZEB1* pathogenic mutations were identified in some studies while not in others showing heterogeneity [41, 42]. *ZEB1* truncating mutations result in reduced protein production and its impaired localization within the cell. Though *ZEB1* function in corneal endothelial cells is unknown, its role in maintaining endothelial cell density and corneal transparency cannot be ruled out [43]. It is hypothesized that *TCF4* expressed in the developing corneal endothelium induces epithelial-to-mesenchymal transition (EMT) and may decrease endothelial cell density [44, 45]. SNPs and repeat expansions may alter the *TCF4* activity and also affect endothelial cell density leading to endothelial dysfunction.

Population structure analysis indicated that the North and South Indians are genetically distinguishable [3]. Considering differences in origin of the two populations, a distinct mutation spectrum would be envisaged, but the studies discussed here prove otherwise. In general, autosomal recessive corneal disorders appear to predominate the patient population of South India. However, the mutation profile is similar for the dystrophies analyzed in both the populations. Overrepresentation of the autosomal recessive disorders reflects high prevalence of consanguinity found in some communities and also suggests that the South Indian population is genetically distinct.

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Genetics of Corneal Endothelial Dystrophies: An Asian Perspective

24

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Abstract

Corneal endothelial dystrophies are a group of disorders marked by dysfunction and loss of the corneal endothelial cells. The three primary endothelial dystrophies are congenital hereditary endothelial dystrophy, posterior polymorphous corneal dystrophy, and Fuchs' endothelial corneal dystrophy. Recent advances in human genetics have led to the discovery of several genes associated with these endothelial dystrophies. In this chapter, we review these corneal endothelial dystrophies with specific emphasis on the molecular genetic studies performed on subjects from Asia.

Keywords

Corneal endothelial dystrophies/endothelial dystrophy • Genetics • Fuchs' endothelial corneal dystrophy • Congenital hereditary endothelial dystrophy • Posterior polymorphous corneal dystrophy

24.1 Introduction

The endothelium is derived from the neural crest during embryonic development as a single layer of cells in a hexagonal mosaic pattern overlying Descemet's membrane. The endothelial cells are arrested in the G1 phase of the cell cycle, and cell density varies from ~ 6000 cells/mm^2 at birth to ~ 3000 cells/mm^2 by age of 5 years [1]. The endothelial cell density decreases throughout the life of an individual. Ethnic differences influence endothelial density. Matsuda et al. compared the endothelium of American whites to age-matched Japanese subjects and identified a

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significantly higher cell density among the Japanese subjects [2].

Descemet's membrane is a thick basement membrane secreted by the endothelium. Ultrastructure of the Descemet's membrane reveals a characteristic banded form of collagen on electron microscopy (anterior banded zone) during fetal development that shifts to a non-banded form (posterior non-banded zone) after birth [3]. When endothelial cells are damaged, they can revert back to the production of the fetal banded collagen resulting in excessive thickening of the Descemet's membrane [3].

Endothelial cells maintain corneal clarity and deturgescence by regulating stromal hydration. The corneal endothelial cells are metabolically active with abundant mitochondria, endoplasmic reticulum, and Golgi complex organelles [4]. This cellular machinery helps in the endothelium's pump function, allowing movement of ions and osmotic withdrawal of water from the stroma into the aqueous humor. The tight junctions between the endothelial cell layers, the zonula occludens, form the endothelial barrier allowing the passage of nutrients and other molecules into the stroma [4].

24.2 Congenital Hereditary Endothelial Dystrophy

Congenital hereditary endothelial dystrophy (CHED) is a rare disorder resulting from dysfunction of the endothelial cells.

An autosomal dominant form of CHED (CHED1, MIM #121700) in a British pedigree has been mapped to chromosome 20 between D20S48 and D20S471 in a region that has also been assigned to posterior polymorphous corneal dystrophy (PPCD) [5]. CHED1 and PPCD may be allelic at this locus as the underlying gene has yet to be identified in this region.

Congenital hereditary endothelial dystrophy (CHED2, MIM # 217700) is an autosomal recessive disorder presenting with bilateral corneal clouding either at birth or during early childhood. Affected members are typically diagnosed at the ages of <1–10 years. Clinical diagnostic criteria for CHED include bilateral cloudy corneas, corneal edema with stromal thickening, and normal intraocular pressures [6].

The degree of corneal opacification in CHED varies from mild to severe including a ground glass or bluish-white appearance. In subjects with severe opacification, nystagmus and amblyopia of varying degrees may develop. The stroma is usually two to three times the normal thickness. Slit-lamp examination of the endothelium is often difficult because of the corneal haze.

A higher incidence of recessive CHED is observed in Middle Eastern countries, India, and Pakistan due to higher rates of consanguinity [7].

Microscopic analysis of corneal tissue in CHED reveals swelling of the stroma, marked thickening of the Descemet's membrane, and endothelial cell atrophy [3]. In CHED, there is abnormal neural crest terminal induction during the later perinatal stages. Failure of complete differentiation of the corneal endothelial monolayer results in dysfunctional endothelial cells and an abnormal Descemet's membrane.

In 1960, a description of congenital hereditary endothelial dystrophy was published by A. Maumenee [8, 9]. Genetic studies linked autosomal recessive CHED to a 20p13 chromosomal region flanked by markers D20S113 and D20S882 [10]. Vithana et al. refined the CHED2 linkage interval to a ~ 2.2 Mb interval containing approximately 30 genes in a Myanmar family with recessive CHED [11]. Vithana et al. screened the Myanmar family for mutations in *SLC4A11* and identified a homozygous mutation in exon 17 resulting in amino acid substitution R775Q [11]. To confirm the role of *SLC4A11* in CHED, Vithana et al. studied 9 additional Pakistan and Indian families with CHED and found *SLC4A11* mutations that co-segregated with the disease in all families and were absent in 50 ethnically matched and 80 Chinese control subjects [11]. Expression of *SLC4A11* in human corneal endothelium was demonstrated by reverse transcription polymerase chain reaction (11). In situ hybridization studies on mouse embryonic eyes demonstrated expression of *SLC4A11* in the cornea at day 18 that corresponded to the fifth month of

human gestation [12, 13]. Studies on HEK293 cells transfected with the mutant and wild-type proteins revealed reduced levels of the mutant proteins indicating that they are not processed by the endoplasmic reticulum and are subsequently degraded [11]. Because of its classification as a solute carrier 4 (SLC4) superfamily, *SLC4A11* was initially assumed to be a borate/bicarbonate transporter. Functional studies in primary bovine corneal endothelial cells, however, identified *SLC4A11* as sodium-coupled hydroxyl ion transporter [14]. Additional *SLC4A11* knockout studies in multiple experimental animal models and human corneas suggest that the ~120 kilo Dalton protein with 14 different transmembrane domains may promote basolateral entry of water molecules into corneal endothelium [14].

Subsequent studies conducted in Indian cohorts identified additional *SLC4A11* mutations in autosomal recessive CHED. Ramprasad et al. identified two nonsense and three missense *SLC4A11* mutations in five consanguineous South Indian families [15]. Another independent study by Hemadevi et al. reported ten additional *SLC4A11* mutations, out of which seven were missense mutations, one nonsense mutation, and two frameshift mutations [16]. Sultana et al. added to the repertoire of *SLC4A11* mutations in South Indian families by identifying 27 different mutations [17]. Paliwal et al. studied the mutational spectrum of *SLC4A11* in a North Indian cohort with a high degree of consanguinity [18]. The study identified one novel and three previously reported homozygous mutations in CHED. In this Indian CHED cohort, electron microscopy studies of specimens revealed a marked increase in the overall thickness of the Descemet's membrane (5–8 times thicker) with a normal thickness anterior banded zone and markedly thickened posterior non-banded collagen zone with attenuated or absent endothelium [18].

In 2013, Kodaganur et al. screened six Indian families with CHED and summarized all the known mutations in *SLC4A11* [19]. Their study identified four novel (p. Thr262Ile, p. Gly417Arg, p. Cys611Arg, and p. His724Asp) mutations and one known p. Arg869His homozygous mutation in the *SLC4A11* gene [19]. A novel nonsense mutation that was not previously reported or found in ethnically matched controls was identified in a Korean CHED case [20]. The novel homozygous mutation c.1239>A in *SLC4A11* resulting in a truncated protein further expands the mutational spectrum of CHED. Approximately 78 different mutations in *SLC4A11* associated with CHED have been reported to date [19].

24.3 Posterior Polymorphous Corneal Dystrophy

Originally described in 1916 by L. Koeppe, posterior polymorphous corneal dystrophy (PPCD) is a rare disorder that is inherited in an autosomal dominant fashion [21, 22]. Slit-lamp examination reveals numerous vesicular or linear band-like lesions at the level of Descemet's membrane. Endothelial changes are often asymmetric with variable levels of decompensation that may result in stromal edema.

The disease pathology involves metaplastic changes to the endothelium resulting in epithelial-like cells invading the posterior cornea and angle structures [23, 24]. These metaplastic cells produce cytokeratins that are typical for epithelial cells [21, 22]. Electron microscopy studies revealed the presence of multilaminar epithelial-like cells joined by desmosomes and nodular excrescences in the Descemet's membrane [22, 23]. PPCD is characterized by anterior banded fetal Descemet's membrane with little to no posterior amorphous Descemet's membrane indicating endothelial dysfunction [24].

PPCD has been linked to three different loci in linkage studies in extended pedigrees: PPCD1 on chromosome 20p11.2-q11.2, PPCD2 on chromosome 1p34.3-p32.2, and PPCD3 on chromosome 10p11.2 [25]. Whereas the gene for PPCD1 is currently unknown, PPCD2 and PPCD3 are attributable to *COL8A2* and *TCF8*, respectively [25].

Linkage studies by Shimizu et al. identified a PPCD locus (PPCD3, MIM # 609141) at an 8.55 cM region of chromosome 10 with >30

genes including *TCF8* [26]. Krafchak et al. discovered that mutations in *TCF8* are responsible for both familial and isolated cases of PPCD [27]. In their study, 5 out of 11 (45%) probands with PPCD had either heterozygous frameshift or nonsense mutations in *TCF8*. This gene encodes for the two-handed zinc finger-homeodomain transcription factor. Krafchak et al. noted that *TCF8* can bind to the promoter of *COL4A3* and provided immunohistochemical evidence of ectopic production of *COL4A3* by the endothelium in a keratoplasty specimen of a PPCD proband [27].

24.4 Fuchs' Endothelial Corneal Dystrophy

In 1910, Ernst Fuchs reported 13 cases of central corneal clouding describing it as "dystrophia epithelialis cornea" [28]. Late-onset Fuchs' endothelial corneal dystrophy (FECD, MIM # 613267) is a progressive bilateral degenerative disorder of middle age characterized by the presence of focal collagenous excrescences of the Descemet's membrane referred to as corneal guttae. The guttae are apparent clinically by slit-lamp microscopy in the central cornea and spread toward the periphery with disease progression. These changes at Descemet's membrane are accompanied by loss of the hexagonal mosaic pattern and decrease of endothelial cell density (Fig. 24.1).

Initially, a person with Fuchs' dystrophy may awaken with blurred vision because of corneal edema that will gradually clear during the day. As the disease progresses, this swelling will remain constant and the vision remains poor throughout the day. Guttae may also result in symptomatic glare. With no proven medical treatments, corneal transplantation is the only treatment option for moderate or advanced disease.

Fuchs' dystrophy affects 1 in 20 Caucasians over the age of 40 in the United States making the corneal disorder the leading indication for corneal transplantation [29]. The prevalence of Fuchs' dystrophy is higher in European countries with its findings manifest in 11% of females and 7% of males in Iceland [30]. The disease is not uncommon in East Asian populations. A higher

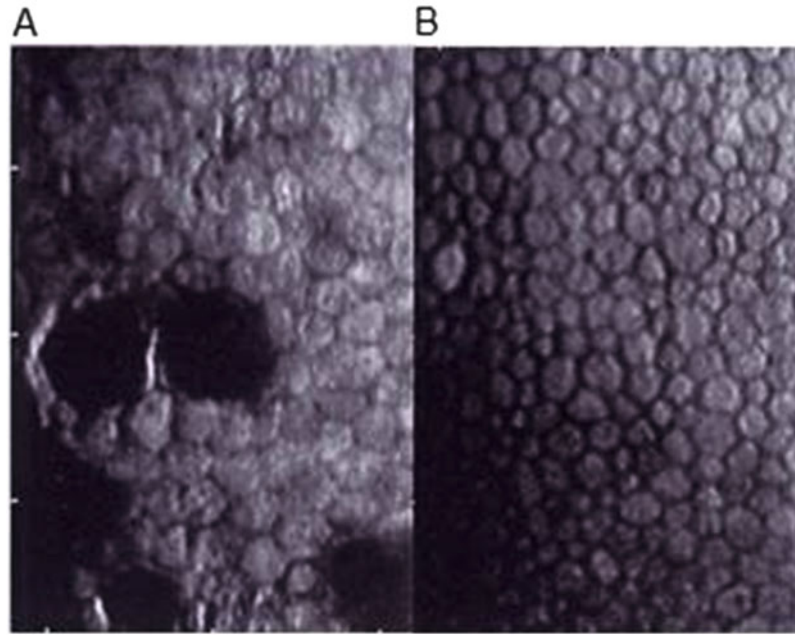

Fig. 24.1 Specular images of endothelium from a subject with Fuchs' dystrophy in **a** compared to an age-matched control in **b**. Note the loss of the hexagonal pattern and decrease in central endothelial cell density in the Fuchs' dystrophy subject. The *dark spots* seen in **a** correspond to the guttae

prevalence of the disease is observed in Singapore relative to Japan (8.5% of Singapore Chinese compared to 5.5% of Japanese) [31].

In 1979, Krachmer et al. proposed a slit-lamp microscopy grading scale ranging from 0 to 6 to document the severity of Fuchs' dystrophy: grade 0, unaffected with no guttae; grade 1, <12 non-confluent guttae; grade 2, >12 non-confluent guttae; grade 3, zone of confluent guttae 1–2 mm wide; grade 4, zone of confluent guttae >2–5 mm wide; grade 5, zone of confluent guttae >5 mm wide; and grade 6, zone of confluent guttae >5 mm wide with corneal edema [32].

Fuchs' dystrophy is characterized by changes in endothelial shape (polymegathism) and size (polymorphism) indicative of premature senescence [33]. Ultrastructure of the Descemet's membrane secreted by endothelial cells has a characteristic banded form of collagen on electron microscopy (anterior banded zone) during fetal development that normally shifts to a non-banded form (posterior non-banded zone) after birth [3]. In Fuchs' dystrophy patients, there is a reversal to the fetal banded form of collagen and diffuse thickening of the Descemet's membrane with focal excrescences [33]. As the guttae enlarge, the endothelial cells become apoptotic leading to stromal edema, scarring, and loss of vision. The loss of endothelial cells in Fuchs' dystrophy is demonstrated by the presence of TUNEL (Tdt-dUTP terminal nick end labeling) positive apoptotic cells [21].

Early-onset Fuchs' endothelial corneal dystrophy (FECD, MIM # 136800) is a rare form of this disorder. Biswas et al. identified a heterozygous *COL8A2* missense mutation in a multigenerational family (Northeast England) with early-onset Fuchs' dystrophy linked to a 7cM region of chromosome 1p34.3-p32 [34]. The point mutation resulted in a lysine to glutamine (Q455K or Gln455Lys) substitution in alpha-2 chain of type VIII collagen. Gottsch et al. identified another point mutation resulting in a leucine to tryptophan substitution (L450W or Leu450Trp) in COL8A2 in an early-onset Fuchs' dystrophy family originally described by Magovern et al. [35, 36]. The findings were present in family members as young as 3 years old. The average age of diagnosis in these families ranged from 21 to 48 years, and by the time the individuals were in their early 40s, they have advanced Fuchs' dystrophy.

Kobayashi et al. screened *COL8A2* in a cohort of Japanese Fuchs' dystrophy patients and identified two missense mutations, R155Q and T502 M. However, these variants were also detected in normal Japanese subjects suggesting that *COL8A2* variants do not play a role in adult-onset Fuchs' dystrophy in this ethnicity [37]. Mok et al. examined six Korean pedigrees with early-onset FECD and found a novel Q455V mutation in COL8A2 gene to be responsible for all the cases [38]. Hemadevi et al. screened 80 Indian patients with Fuchs' dystrophy and 100 age-matched controls for mutations in *SLC4A11* and *COL8A2* [7]. The study identified two novel mutations in *COL8A2* and *SLC4A11* in both Fuchs' dystrophy and control subjects, concluding that these two genes do not play a significant role in their Indian Fuchs' dystrophy cohort.

Mutations in *TCF8* also referred to as *ZEB1* (zinc finger E-box binding homoebox 1) may play a role in late-onset FECD. In a Singapore-Chinese cohort, Mehta et al. screened 74 Fuchs' dystrophy subjects with 8 familial and 66 sporadic cases and identified a novel p. N696S variant. The presence of only one variant implied that *TCF8* does not play a major role in the pathogenesis of Fuchs' dystrophy in their Chinese cohort [39]. However, Rizauddin et al. identified five novel missense mutations in *TCF8* in a North European cohort of 192 late-onset Fuchs' dystrophy patients which were absent in ethnically matched controls [40].

Vithana et al. reported the association between *SLC4A11* mutations and late-onset Fuchs' dystrophy [41]. In their study 89 late-onset Fuchs' dystrophy subjects (of which 64 individuals were Chinese from Singapore and Hong Kong), four novel heterozygous *SLC4A11* mutations were identified that were absent from the age-matched controls. According to this study, ~5% of Fuchs' cases can be attributed to *SLC4A11* mutations in a Chinese cohort. The missense mutations (T754M,

G709E, and E399K) resulted in aberrant glycosylation and decreased S*LC4A11* protein levels [41]. Rizauddin et al. screened a cohort of 192 subjects with late-onset Fuchs' cohort and identified 7 novel *SLC4A11* mutations that were absent from ethnically matched controls [42]. Collectively, these studies suggested that heterozygous mutations in *SLC4A11* contribute modestly to the pathogenesis of late-onset Fuchs' dystrophy.

In 2010, a genome-wide association study demonstrated the strong association of single nucleotide polymorphisms (SNPs) in the *TCF4* (transcription factor 4) gene and Fuchs' dystrophy in white subjects [43]. One copy of the intronic SNP rs61387 risk allele conferred a 5.47-fold increased risk for disease. *TCF4*, a member of the E-protein family, is a ubiquitous basic helix-loop-helix (bHLH) protein and is thought to play an important role in epithelial-mesenchymal transition [43].

A study by Thalamuthu et al. in 57 Chinese subjects with Fuchs' dystrophy and 121 control subjects demonstrated that the intronic SNP rs613872 was not polymorphic in the Chinese cohort [44]. Instead, two other *TCF4* SNPs which were ~2000 base pairs away, rs17089887 and rs17089925, showed strong association with the disorder in the Chinese cohort.

In 2012, Wieben et al. demonstrated a strong association between an intronic CTG trinucleotide repeat expansion (CTG18.1) in *TCF4* and Fuchs' dystrophy [45]. In their study, 52 out of 66 white FECD subjects had greater than 50 CTG repeats at this locus as compared to 2 out of 63 control subjects. This group reported the triplet repeat expansion to be a better predictor of disease than rs613872 and hypothesized that the repeat polymorphism was a likely functional variant. A subsequent study by Mootha et al. with 120 FECD white patients and 100 control subjects demonstrated that one copy of the CTG18.1 expansion of greater than 40 CTG repeats increased the risk of disease by 32.3-fold [46]. The rs61387 SNP was found to be in linkage disequilibrium (LD) with the CTG18.1 locus in this Caucasian cohort. This study documented that the expanded CTG18.1 allele co-segregated with the disease trait with complete penetrance in 52% of 29 families and with incomplete penetrance in additional 10% of families. In a trans-ethnic association study, the CTG18.1 expansion was found to be strongly associated with Fuchs' dystrophy in a Singapore-Chinese FECD cohort conferring a 66.5-fold increased risk for disease [47]. To examine the linkage disequilibrium structure of *TCF4*, a haplotype analysis was performed in this Chinese cohort and compared with the 97 Han Chinese and 85 Caucasians using data from the 1000 Genomes Project. The only haplotype associated with the disease phenotype was the one with the CTG18.1 repeat expansion. Based on these trans-ethnic studies, it was concluded that the expanded CTG18.1 allele is a common, functional variant for FECD in Eurasian populations [47].

In a study of 44 subjects with FECD from Bhubaneswar in South India, the *TCF4* SNP rs17089887 was found to be strongly associated with Fuchs' dystrophy, but there was no association with rs613872 or rs17089925. This group also found a strong association of the CTG18.1 expanded allele and FECD with 34% of subjects harboring the expansion compared to 5% of controls. Nakano et al. studied the association between CTG18.1 repeat expansion and Fuchs' dystrophy in 47 patients and 96 controls in a Japanese cohort [49]. The study reported triplet repeat expansions in 26% of Fuchs' dystrophy patients and 0% of the controls.

Oxidative stress-induced apoptosis contributes to the disease pathology in Fuchs' dystrophy [48]. Corneal endothelial apoptosis has been demonstrated in Fuchs' dystrophy by TUNEL staining (terminal deoxynucleotidyl transferase dUTP nick end labeling) and DNA fragmentation assays [32]. Corneal endothelial cells are arrested in G1 cell cycle phase and are highly susceptible to reactive oxygen species-induced apoptosis [50]. Proteomic analysis of the corneal endothelial cells from normal and Fuchs' dystrophy subjects revealed increased levels of advanced glycation end products in the Fuchs' dystrophy samples [51]. Jurkunas et al. further demonstrated that there is an

oxidant-antioxidant imbalance in Fuchs' dystrophy which in turn leads to oxidative DNA damage [52].

In 2015, two reports documented the accumulation of CUG repeat transcripts as ribonuclear foci in the corneal endothelium of subjects with CTG18.1 expansions in *TCF4* [53, 54]. Previously, RNA nuclear foci had been only reported in rare neurodegenerative disorders mediated by simple repeat expansions [55]. Du et al. reported that the tissue-specific splicing factor, muscleblind-like protein 1 (MBNL-1) co-localizes with the CUG repeat foci and provided some evidence of alternate splicing of genes mediated by MBNL1 [53]. Results from both independent studies implicate a role of gain of function RNA in the molecular pathogenesis of Fuchs' endothelial corneal dystrophy in subjects with the *TCF4* triplet repeat expansions.

24.5 Summary

Congenital hereditary endothelial corneal dystrophy, posterior polymorphous corneal dystrophy, and Fuchs' endothelial corneal dystrophy are a group of inherited disorders of the endothelium. In these related disorders, dysfunction and attrition of the endothelial cells result in loss of corneal clarity and decreased vision. With no proven medical treatments, keratoplasty is often required to rehabilitate vision in subjects with these corneal dystrophies. Asiatic studies have been particularly relevant in elucidating the genetics of congenital hereditary endothelial dystrophy and Fuchs' endothelial corneal dystrophy. A better understanding of the molecular genetic basis of these corneal dystrophies may lead to improved clinical diagnosis and development of molecular treatments in the future.

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Keratoconus in Asia

25

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Abstract

Keratoconus (KC) is a common slow-progressing corneal dystrophy characterized by stromal thinning and astigmatism that cause loss of visual acuity. The clinical symptoms vary greatly, from a mild subclinical stage to increasing severity involving conical protrusion, thinning of the cone apex, irregular astigmatism, myopia followed by central scarring, and perforation at a later time leading to loss of vision. The precise molecular mechanism that mediates keratoconus is still unknown. Several genetic and nongenetic factors such as frequent eye rubbing, excessive ultraviolet light exposure, or recurrent allergies have been associated with causes of keratoconus. The progression of disease involves biochemical, structural, and cellular changes caused by modifications in oxidative stress, corneal collagens, inflammatory mediators, matrix metalloproteinase enzymes, and epithelial-stromal proteins. The Mendelian inheritance patterns suggest the role of genetics in this disease. At present there are no potent therapies available that treat, cure, or arrest the progression of keratoconus disease except surgical intervention or crosslinking procedures. This chapter discusses the current status of keratoconus epidemiology, pathology, genetics, and molecular understanding.

Keywords

Keratoconus • Corneal dystrophy • Single-nucleotide polymorphisms • IL1B • CDH11 • NUB1 • COL27A1, and HGF • RAB3GAP1, and LOX

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25.1 Introduction

In keratoconus (KC), a corneal disease characterized by vision distortion resulting from irregular astigmatism, progressive stromal thinning and ectasia occur [1, 2]. The clinical features of this disease range from the "forme frusta," a mild, subclinical stage, to conical protrusion of increasing severity, thinning of the cone apex, irregular astigmatism, myopia, and later-stage complications of central scarring, hydrops, perforation, or blindness [2, 3]. This disease is complex and involves various factors of both genetic and environmental sources that contribute to the disease pathology. Because keratoconus is a multifactorial disease, it is often challenging to establish the correct etiology. Only recently have we begun to understand the genetic and molecular factors driving this disease. Lack of appropriate animal models for studying the disease also makes scientific understanding of the disease more tedious.

Clinically, the keratoconus pathology is typically assessed by topographic and biomechanical parameters [4, 5], but tissue factors driving ectasia are not fully understood. Studies have demonstrated a link of keratoconus with atopy, hard contact lens usage, eye rubbing, and inflammatory factors [6–8]. Progression of keratoconus could be related to conditions of allergic etiology [9, 10] or higher systemic oxidative stress [11, 12]. Recent reports state that matrix-degrading enzymes (MMPs) and inflammatory cytokines [7, 9, 13, 14] drive the pathogenesis of the disease. Studies investigating biochemical and pathological changes [15–17] at the structural and cellular level [18] are as yet not conclusive about the underlying molecular mechanisms of keratoconus or the influence of the local biochemical environment. In keratoconus [17], collagen content and types do not vary appreciably [15], despite the significant structural changes within the corneal stroma. Hence, the ectatic stress, and the physical and inflammatory milieu, have been suggested to induce the deregulation of numerous stromal and epithelial proteins that ultimately precipitate the disease [12, 19, 20]. It is therefore thought that various environmental and other stimuli exacerbate a genetic predisposition leading to the disease pathology [21]. Hence, the causative factors driving ectasia may be underlying genetic defects with Mendelian inheritance patterns [22], which concept is also supported by evidence for ethnic differences observed in keratoconus [23]. Some studies suggest consanguinity as a risk factor for keratoconus [24], thereby arguing in favour of a greater role for genetic mechanisms in this disease. Currently, there are no drugs or therapies available to halt the progression of the disease, thereby necessitating surgical intervention or crosslinking procedures [25, 26]. This chapter attempts to accrue the current knowledge on genetics of keratoconus that may have bearing on a greater understanding of the molecular basis of disease progression.

25.2 Keratoconus Epidemiology

Keratoconus is prevalent across the globe, but genetic variations and different diagnostic criteria may be responsible for differential prevalence data in given populations [2, 3]. The estimated incidence of keratoconus varies between 1 in 500 and 1 in 2000 individuals in the general population, and the estimated prevalence is reported to be 54.5 per 100,000 [2, 27–29]. This number has been challenged by the reduced numbers reported in additional population-based studies [23, 30], indicating that a systematic epidemiological study of keratoconus incidence using uniform diagnosis criteria requires implementation. The incidence rate in the Caucasian population has been reported to be lower than in Asian populations by as much as 7.5 fold [23, 31], as demonstrated by cross-ethnic studies in the United Kingdom. Studies indicate that in Asian ethnicities such as Middle Easterners, Indians, Pakistanis, and Polynesians, the disease is not only more prevalent but may also have early onset and faster progression [23, 31, 32]. A similar increased incidence in Indian and Pakistani patients has

been reported in Singapore [33]. The incidence of keratoconus in Middle Eastern countries such as Saudi Arabia and Iran is reported to be between 20 in 100,000 and 24.9 in 100,000 [34, 35], and the incidence is 7.6 in 100,000 in the Japanese [36]. Interestingly, a few studies have observed mitochondrial haplogroups associated with keratoconus in Arab patients [37, 38]. Prevalence studies in Western India report keratoconus to be fairly high at 2.3% (2300 per 100,000) [39, 40], although a cutoff power ≥49 Diopter was applied. A similarly high prevalence of 2.34% is reported in the young, college-going population in Jerusalem [41]. The observed ethnic differences may stem from the geographic location: India, Pakistan, and the Middle East all are hot countries with more exposure to sun compared to the USA, European countries, or Japan. In addition, consanguinity [42] is a prevalent practice in these countries, which may lead to a greater prevalence of inherited diseases.

25.3 Disease Pathology and Clinical Grading Criteria

The screening of keratoconus involves keratoconus-related clinical signs such as retinoscopy scissors reflex, Munson sign, stromal thinning, Vogt's striae, and Fleischer's ring, but corneal topography is the most useful method in the diagnosis of keratoconus, especially in the absence of clinical signs. Several devices are currently available for detecting early keratoconus by measuring anterior and posterior corneal topography and elevation [43, 44]. Corneal topographic and tomographic techniques, which generate color-coded maps and topographic indices, are the most sensitive devices for confirming the diagnosis of keratoconus [2, 45]. In addition, videokeratography has been shown to identify forme fruste keratoconus (FFKC) in the absence of clinical signs of keratoconus. Videokeratographic indices such as the Klyce–Maeda criteria, the Rabinowitz criteria, and others have been developed to quantitatively analyze videokeratography and to screen for keratoconus [45]. These indices have been shown to identify keratoconus with a high degree of sensitivity and specificity. The Orbscan II is a three-dimensional slit-scan topography system for analysis of the corneal surfaces and anterior chamber and uses calibrated video and a scanning slit beam to measure x, y, and z locations of several thousand points of the patient cornea. These points are used to construct topographic maps [45]. The Pentacam (Oculus Inc.) is a corneal tomography technology that generates data on topography and the elevation of the anterior and posterior using a rotating Scheimpflug camera that generates a topographic map. Various diagnostic parameters are available for keratoconus diagnosis depending on the mode of topography that is being used. Maeda and Klyce designed a system to detect keratoconus: this system, which is based on linear discriminant analysis and a binary decision tree, identifies the map as representing keratoconus or non-keratoconus and based on a value from the discriminant analysis (the KPI), assigns the map an index expressed as a percentage that suggests the severity of keratoconus. At this time, however, the Keratoconus Severity Index (KSI) and the Amsler–Krumeich classification are the most popular methods for grading keratoconus severity [43, 44]. KSI is based on indices estimated by a curvature map using Placido disk-based corneal topography, and the Amsler–Krumeich classification defines the stage of keratoconus using biomicroscopy, mean central keratometry reading, spherical and cylindrical refraction change, and corneal thickness [43, 44].

25.4 Genetics of Keratoconus in Candidate Genes

Keratoconus is a complex heterogeneous progressive disorder with multifactorial etiology, although it has been shown that genetic factors were associated with increased incidence in monozygotic twins and familial inheritance [2, 46, 47]. Most of the reported keratoconus patients are isolated, and 6–10% of patients

showed a family history [48, 49]. So far keratoconus appears in a dominant and recessive inheritance pattern, but incomplete penetrance with a variable phenotype has been observed in an autosomal dominant condition [49]. Several genomic loci were associated with keratoconus families in various ethnic origins, which suggests genetic heterogeneity [22, 50–53]. During the embryonic development, VSX1 (a member of the paired-like homeodomain transcription factors) might regulate the expression of cone opsin genes and retinal bipolar interneurons [54–56]. Several missense coding variations in the *VSX1* gene were found to be associated with keratoconus patients from various ethnic origins [57–62]. Interestingly, the *VSX1* variant (L268H) shared with common single-nucleotide polymorphisms (SNPs) (haplotype) found in two unrelated Indian families with keratoconus suggests the possibility of a founder effect [62]. The coding variant of p.Gln175His in the homeodomain of VSX1 showed in an Indian family of keratoconus with incomplete penetrance [60]. A large number of unrelated Indian patient cohorts with keratoconus showed the absence of *VSX*1 mutations, suggesting the involvement of other factors such as multiple genes and environmental interaction in the disease pathogenesis [63]. A study on VSX1 expression in mouse and human cornea revealed that there was no expression detected by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis [64]. In addition, mice models of $VSX1^{+/-}$ and $VSX1^{-/-}$ did not show the typical keratoconus phenotype [63]. So far, few keratoconus patients have been associated with *VSX1* mutations, suggesting that these might not be involved in the pathogenesis of keratoconus [65, 66]. A possible candidate gene dedicator of cytokinesis 9 (*DOCK9*) encodes the DOCK protein family that belongs to GTP/GDP exchange factor activity and specifically activates G-protein CDC42, which is expressed in keratoconic and non-keratoconic corneas; moreover, three different missense coding variants were found in screening of eight candidate genes within the 13q32 locus [67]. Growing evidence suggests that oxidative stress has been implicated as involved in corneal diseases [68]. *SOD1*, a major cytoplasmic antioxidant enzyme, metabolizes superoxide radicals and protects against oxygen toxicity [68]. In familial keratoconus patients, a 7-bp deletion at intron 2 of the *SOD1* gene was found that results in loss of the active site in the SOD1 enzyme [69]. The increased copy numbers of mitochondrial DNA content associated with patients with keratoconus may indicate mitochondrial respiratory chain defects or the involvement of mitochondrial abnormality [70, 71]. Several recent studies suggest increasing levels of mitochondrial DNA damage and cytotoxic by-products in keratoconic corneas [68, 72]. The precise role of *SOD1* in the pathogenesis of keratoconus has not been fully understood, as *SOD1* gene mutations were identified in patients with familial amyotrophic lateral sclerosis (ALS) with no signs of corneal abnormalities or keratoconus phenotype [69, 73]. A transcription factor, ZEB1, is involved in the epithelial-to-mesenchymal transition (EMT). *ZEB1* mutations were found in keratoconus patients and isolated patients of posterior polymorphous corneal dystrophy (PPCD), which might indicate the possible genetic association of *ZEB1* in keratoconus and other corneal diseases [74, 75]. Recently, a micro-RNA-184 (miR-184) mutation altering the seed region was identified in familial keratoconus patients with early-onset anterior polar cataract [76]. The keratocyte apoptosis was observed in the keratoconic cornea, which showed that apoptotic processes are a part of the pathogenesis [77]. Filaggrin (*FLG*) is an apoptosis-related gene, expressed in human corneal epithelium: mutations in *FLG* were associated with atopic dermatitis. In five keratoconus patients, loss of functional FLG protein (R501X and 2282del4) as a result of nonsense mutations suggests a role of *FLG* in keratoconus pathogenesis [78]. Another hypothesis is that keratoconus pathogenesis may be caused by the changes in corneal collagen structure. Nevertheless, mutation analysis of *COL4A3* and *COL4A4* in patients with keratoconus revealed no pathogenic changes, however, significant allele frequency was observed in

COL4A3 (D326Y) and *COL4A4 (*M1237 V, F1644F) variants in keratoconus patients [79]. Another genetic study on fifteen Ecuadorian families with keratoconus identified sequence variations in *COL4A1* and *COL4A2*, but none of these segregated with affected family members [80]. No pathogenic mutations were identified in the *COL8A1* and *COL8A2* genes in keratoconus patients [81]. The gene *ZNF469* encodes a zinc-finger protein and may function as a transcription factor or extranuclear regulator factor for the synthesis and organization of corneal collagen fibers. Mutations in this gene cause keratoconus and brittle cornea syndrome [82, 83]. A gene called transforming growth factor beta-induced (*TGFβI*), responsible for several dominant corneal dystrophies, is a cytokine shown to be an important regulator for extracellular matrix formation during the repair and injury of tissue. A novel stop codon mutation G535X in *TGFβI* was found in a keratoconus patient of Chinese origin. Additionally, a heterozygous missense mutation (Arg533Gln) was detected in Polish patients with keratoconus [84]. In corneal fibrosis, TGFβ1 pathway markers have a major role and were shown to be increased in advanced cases of keratoconus [85, 86]. In contrast, a mouse model that lacked TGFβ1 showed normal development in the eye [87]. No pathogenic variants were identified in *LOX* or the *TIMP3* gene in large cohorts of Italian patients with keratoconus [88].

25.5 Keratoconus and Genome-Wide Association Studies

Because the genetic etiology of keratoconus is complex, genome-wide association studies (GWAS) might be a useful method to identify single-nucleotide polymorphisms (SNPs). The frequency of SNPs significantly differs between patients and normal individuals, which may help to assess the risk of disease [89]. GWAS on keratoconus patients revealed a few possible candidate genes including *IL1B*, *CDH11*, *NUB1*, *COL27A1*, and *HGF*, *RAB3GAP1*, and *LOX*. These genes were associated with a risk factor for the development of keratoconus. It has been reported that 60% of keratoconic corneas showed keratocyte apoptosis, which might be regulated or triggered by corneal epithelial cell-releasing interleukin-1 (*IL1*) [90–93]. Li et al. found a SNP in GTPase-activating protein subunit 1 (*RAB3GAP1*) that showed as a susceptibility locus for keratoconus in Caucasian patients [67]. In response to corneal injury of corneal keratinocytes, hepatocyte growth factor (HGF) expression was upregulated, which binding site for cytokine IL-6 is elevated in keratoconus patients [94, 95]. Recently, Lu and colleagues found multiple loci associated with central corneal thickness (CCT) and keratoconus [96]. Further, this study showed that two CCT-associated loci, *FOXO1* and *FNDC3B*, conferred relatively large risk factors in two cohorts with 874 cases and 6085 controls [96]. A substitution at *IL1RN* and a deletion at *SLC4A11* were identified using nonparametric linkage analysis in Ecuadorian families with keratoconus [97]. IL1RN is a modulator of inflammatory response that belongs to a cytokine family, a variety of coding variants in *SLC4A11* associated with Fuchs endothelial corneal dystrophy (FECD) and corneal endothelial dystrophy (CHED2) [98].

Genome-wide association studies (GWAS) on keratoconus patients showed that the strongest association of risk factor allele (rs3735520) is near the *HGF* gene; additionally, *LOX* (rs2956540) alleles have a protective effect. Both these SNPs may serve as genetic risk markers for keratoconus patients of European origin [99]. In addition, HGF was shown to have a significant association (rs2286194) in Australian populations [100]. A study on *COL4A4* gene polymorphisms and association of Iranian patients with keratoconus revealed that SNPs (rs2229813) have a role as risk factors for developing keratoconus in this population [101]. Interestingly, mitochondrial haplogroups H and R and mtDNA mutation were shown to be an increased risk factor to develop keratoconus in Saudi Arabian patients [37, 38]. However, in Han Chinese patients, the mitochondrial copy number rather than a haplotype was found associated with keratoconus [71]. Endonuclease 1 (FEN1) has a major function in base excision repair

during oxidative DNA damage; the T/T genotype of the *FEN1*(g.61564299G>T) polymorphism showed a significant association with the risk of keratoconus in patients from Poland [102]. In the same population, it was found that the T/T-G/A combined genotype of *FAS* (rs1800682) and *FASLG* (rs763110) genes may constitute an increased risk of keratoconus occurrence [103]. Another type of base excision repair (BER) genes involved in DNA damage by oxidative stress, the A/A genotype of the c.–1370 T>A polymorphism of the DNA polymerase-γ (*POLG*) gene and A/G genotype and the A allele of the c.1196A>G polymorphism of the X-ray-repair cross-complementing group 1 (*XRCC1*), were associated with increased occurrence of keratoconus. In contrast, the A/T genotype of (*POLG*) and G/G genotype and the G allele (*XRCC1*) were shown to decrease keratoconus risk [104]. Synowiec et al. recently observed that polymorphisms in the *LIG3* genes (g.29661G>A and g.29059C>T) may be involved in keratoconus and that FECD pathogenesis might be a disease marker in keratoconus patients of Polish origin [105]. A case-control study on base excision repair gene APEX nuclease 1 (*APEX1*) showed that the G/T and T/T genotypes of the c.−468 T>G polymorphism were associated with a decreased occurrence of keratoconus and an increased occurrence of keratoconus, respectively [106]. Central corneal thickness (CCT) exome-wide association analysis revealed that the exonic variant of *WNT10A* (rs121908120) was shown as a risk factor of keratoconus in patients from Western Australia [107].

25.6 Asian Perspective of Keratoconus Genetics

A genetic risk factor, the visual system homeobox 1 (*VSX1*) gene, was observed in 1.6% of Indian keratoconus patients ; in fact, incomplete penetrance of the *VSX1* pathogenic variant was showed in a keratoconus family [60, 108]. Surprisingly, coincidental selection of study population or keratoconus families belonging to an endogamous community of origin showed a high frequency of *VSX1* mutations (25%) and founder effect [62]. In contrast, *VSX1* mutations were absent in isolated cases of keratoconus, showing the genetic complexity and controversial involvement of the *VSX1* gene [63, 109]. Mutations in the aryl hydrocarbon receptor-interacting protein-like 1 (*AIPL1*) gene were associated with Leber's congenital amaurosis (LCA) and anterior keratoconus in Pakistani families [53, 110, 111]. Two missense mutations in the *VSX1* gene (p.R166W and p.H244R), which cosegregate in familial keratoconus, might have possible pathogenic influences on KTCN [59]. In southwest Iran, the missense variant H244R was found in a patient and also in healthy control individuals [112]. A keratoconus patient cohort from Saudi Arabia revealed no pathogenic *VSX1* mutations [113]. Although 5.3% of *VSX1* coding variants were reported in keratoconus patients from South Korea in one study, no association was reported in another case-control study [58, 114]. A missense substitution of *VSX1* (p.D144E) was found in an Ashkenazi Jewish family with keratoconus; carriers of this mutation were manifested with pathological corneal findings [115]. Two point mutations and a polymorphism were indentified in the *TGFBI* gene in a Chinese population [116]. Although no pathogenic mutations were found in the *SOD1* gene in Saudi and Iranian cohorts of keratoconus, another study reported that an intronic deletion was associated with familial keratoconus [59, 69, 117]. A total of 84 nucleotide variations were found in the mitochondrial complex 1 gene from Indian patients with keratoconus [118]. Mitochondrial haplogroups H and R were observed in keratoconus patients from Saudi Arabia, but in a Han Chinese population of keratoconus patients it remained unassociated [38, 71]. Two independent studies reported that *IL1B* promoter polymorphism was strongly associated in Korean and Japanese patients with keratoconus [90, 119]. Genome-wide association analyses in European and Asian populations revealed multiple genetic loci associated with central corneal thickness and keratoconus [96]. Tag single-nucleotide polymorphisms (tSNPs) of *VSX1* and *IL1A* genes were associated with a high risk of keratoconus in a Han Chinese population [120]. The first

genome-wide survey of corneal curvature, which is implicated in keratoconus, has been associated with variants in *FRAP1* and *PDGFRA* in Asian populations from Singapore [121]. *FOXO1* and *FNDC3B* genes were associated with large risks for keratoconus patients from Asian and European origins [96]. The evaluation of a possible relationship between *COL4A4* (rs2229813) and LOX (rs1800449) genes in keratoconus patients from an Iranian population showed that the polymorphisms were risk factors for developing keratoconus [101, 122]. In a Han Chinese population, the association between keratoconus and reported genetic loci suggests that rs1324183 (MPDZ-NF1B) is a common genetic risk for keratoconus and requires further investigation [123].

25.7 Summary

The studies discussed here demonstrate the large variation in the genetic landscape of keratoconus. The ethnic variation in prevalence strongly supports a genetic or epigenetic component, or both, in disease etiology. However, the keratoconus genetic studies thus far might have been hampered by small sample sizes and catchment areas, and variation in clinical presentation and diagnosis, leading to heterogeneity in gene association reports. In summary, large-scale multicenter genomic studies should be conducted, particularly in South Asia, to establish the genetic predisposition profile of the keratoconus patient (Tables 25.1 and 25.2).

Table 25.1 Genetic studies on keratoconus patients from the worldwide population

Genes	Functions	Population	References
VSX1	Ocular development and craniofacial	European, Italian	[61, 65, 84, 124]
SOD1	Antioxidant enzyme that metabolizes superoxide radicals and provides a defense against oxygen toxicity (oxidative stress)	Americans, Greek	[69, 125]
ZEB1	Involved in modulation of epithelial-to-mesenchymal transition (EMT)	Northern European	[126]
TGFBI	It is a cytokine with a role in tissue injury and repair that interacts with an extracellular matrix protein	Polish	[84]
MIR184	Expressed in the cornea and lens, 3′-UTR of two target genes, INPPL1 (inositol polyphosphate phosphatase-like 1) and ITGB4 (integrin beta 4); these two target genes are involved in corneal healing after injury	Australian, British, Northern Irish	[76, 127]
DOCK9	Activators of small G proteins involved in intracellular signalling networks expressed in corneal epithelium	Polish, Ecuadorian	[67, 84]
COL4A3/*COL4A4*/*COL5A1*	Corneal collagen structure, function and/or development during embryology	European, Americans	[79, 128]
FLG	Apoptosis-related, genetic risk factor for atopic dermatitis, with the protein expressed in the corneal epithelium	French	[78]
CAST	Natural endogenous highly specific inhibitor of calpains, calcium-dependent cysteine proteases; influence many aspects of cell physiology including migration, proliferation, and apoptosis	Americans	[129]
HGF	Regulates cell growth, cell motility during wound healing in epithelial cells	Australian, Americans, Europeans	[92, 99, 100]
RAB3GAP1	Catalytic subunit of a GTPase-activating protein that has specificity for Rab3 subfamily, required for normal eye and brain development	Americans, Australian	[130, 131]
LOX	A copper-dependent amine oxidase responsible for the development of lysine-derived crosslinks in extracellular matrix proteins, such as collagen and elastin	Americans	[132]

Table 25.2 Studies of genes associated with keratoconus patients from Asian origins

Genes	Study cohorts	Methods	Origin	References
VSX1	Case-control, familial	Candidate gene	Indian, Iranian, Korean, Ashkenazi Jewish	[58–60, 62, 108, 109, 112, 114, 115]
TGBI	Case-control	Candidate gene	Chinese	[116]
IL1A	Case-control	Candidate gene	Korean, Chinese	[90, 120]
IL1B	Case-control	Candidate gene	Korean, Japanese	[90, 119]
FOXO1, FNDC3B	Case-control	GWAS	Asian	[96]
FRAP1, *PDGFRA*	–	GWAS	Asian Indians, Chinese	[121]
MPDZ-NF1B	Case-control	GWAS	Han Chinese	[123]

GWAS genome-wide associated study

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Genetics of Pediatric Eye Diseases and Strabismus in Asia

26

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Abstract

With increasing emphasis on genetics as a direct cause, susceptibility modulator, or an association of most diseases affecting mankind, there has been generation of great interest in finding out the genetic determinants of the same. Asia represents a continent, a geographical distribution of landmass, populated by many different ethnic populations, namely, Caucasians, East Asians, South Asians, and so forth. As essentially Asia, Africa, and Europe represent the "old world," ancient transmigrations of populations and interethnic marriages have led to a significant genetic overlap modified by epigenetic factors. Subsequently, it was this relatively "mixed" population that transmigrated to the supposed "new world" or landmasses that had isolated, pure, and indigenous populations, and the story was repeated again. Thus, currently one may expect a certain global homogeneity of genetic determinants for most diseases affecting mankind, which are, however, still modulated locally by significant environmental, social, and other epigenetic factors. This is especially so because inheritance patterns show familial repeatability, thus suggesting the presence of some form of genetic transmission, but do not always fit Mendelian patterns. They have thus been aptly included under the umbrella of being "complex" genetic disorders. With increasing cost-effective availability of better tools for genetic analysis, the disease and the search for its genetic origin have both now entered a new era of scientific query. This chapter specifically addresses the current status of genetics of eye disorders in children including strabismus, in the continent of Asia, comprising many parts, i.e., Central Asia, South Asia, Southeast Asia, East Asia, and more and a comparison with the current status in the Western world.

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26.1 Introduction

Pediatric eye disease is a generic term. Almost any condition that is seen in adults may be seen in children. Also, adults may actually manifest disorders that originated in childhood. Again, strabismus may be seen in both children and adults. Common forms of primary strabismus like infantile esotropia (IEs), intermittent exotropia (IEx), and accommodative esotropia (AE) show familial patterns, but many conditions like IEx may actually manifest as constant strabismus only in adulthood, especially if not managed properly in childhood [1]. However, there may be other causes of strabismus acquired in adulthood like cranial nerve palsies, age-related degeneration of connective tissue supporting the extraocular muscles (EOMs), neuromuscular problems, trauma, and others, which are secondary to well-defined clinical etiologies and where genetics is primarily not expected to be the cause for the condition, either directly or by association [2, 3]. These will not be included in this review, which thus aims to be a synopsis of research done for monogenetic eye disorders primarily originating in childhood, including inborn forms of strabismus.

26.2 Epidemiology

There are very few large epidemiological studies on genetics of different eye disorders in Asia. A large overview was performed in 1987 in Shanghai, China, but as this was before the major modern genetic tools were available, it is largely an epidemiological study [4]. Mass screening covering more than 700,000 people was conducted, and about 5000 pedigrees of genetic eye diseases were collected and analyzed all over China. Based on this large genetic study conducted by the Section of Ophthalmic Genetics of the Chinese Society in China, the prevalence and mode of inheritance of dyschromatopsia (4.89%), degenerative myopia (0.95%), retinitis pigmentosa (0.03%), congenital ptosis (0.18%), congenital microphthalmos (0.009%), congenital cataract (0.037%), congenital glaucoma (0.004%), corneal dystrophy, Leber hereditary optic neuropathy LHON, congenital nystagmus (0.025%), coloboma of the eye (0.007%), aniridia (0.00075%), retinoblastoma, macular dystrophy, simple myopia, primary glaucoma, and strabismus in the Chinese population were established. Though this study was not specific for the pediatric age group, most of the conditions assessed are usually seen in children. In 1989 a report was published on 3490 children in north India, where it was demonstrated that close to 50% of conditions were inherited comprising refractive errors, squint, and congenital nasolacrimal duct obstruction [5]. A Japanese study on X-linked ocular albinism (OA) described the spectrum of the disease in the Japanese population. This comprised a wide range of ocular manifestations including nystagmus, macular hypoplasia, posterior embryotoxon, megalocornea, and absence of stereopsis, to almost normal-looking eyes consisting of a non-albinotic fundus with moderate pigmentation, good visual acuity, stereopsis, and brown non-translucent irides [6]. An Indian study on the severity of isolated retinitis pigmentosa (RP) in 134 subjects demonstrated autosomal recessive inheritance in 72%, autosomal dominant inheritance in 17%, and X-linked inheritance in 11%. Legal blindness was noted in 50% of subjects demonstrating AR or X-linked recessive pattern RP and 32% AD pattern RP. The study did not elaborate on the significance of this difference [7]. Comparison with primarily Caucasian database is elaborated in Table 26.1.

A recent Indian study on the co-segregation of mutations in gamma crystalline A and B genes in

Table 26.1 Etiopathogenesis and genetic aspects of pediatric eye disorders and strabismus

S. No	Condition	Etiopathogenesis	Clinical features	Genetic aspects of reviewed Asian studies	Affected genes in other ethnic populations	Comments
1	X-linked congenital Nystagmus [36–44]	Primarily familial in origin	Conjugate, right-beating at right gaze, left-beating at left gaze, plane of nystagmus independent of direction of gaze, dampening on convergence	FRMD7 mutations X-linked recessive (XLR) inheritance	FRMD7 gene domain XLR inheritance	Only one gene identified for the X-linked form in the Asian and other ethnic populations
2	Albinism [47, 50] Oculocutaneous (OCA) and ocular albinism (OA)	Deficiency in melanin-producing enzymes or in melanin distribution	Hypopigmentation of the skin, iris, fundus, foveal hypoplasia, typically idiopathic/sensory defect nystagmus	Multiple genes affected (TYR, OCA2, TYRP1, SLC45A2, SLC24A5, SILV) GPR143	Multiple genes affected (TYR, OCA2, TYRP1, SLC45A2, SLC24A5, SILV) GPR143	Same genes affected in the Asian and other ethnic populations
3	PMD and other dysmyelinating conditions [10, 51, 52]	Inability to form myelin Leukodystrophy	Hypotony, nystagmus, spasticity, ataxia, choreoathetoid movements, intellectual disability (ID)	PLP1; XLR inheritance	PLP1; XLR inheritance	Same gene affected in the Asian and other ethnic populations
4	Pediatric cataract [8, 17, 53, 54]	Multifactorial; metabolic causes are a primary cause; can be associated with infectious and inflammatory etiology; as also familial forms are observed	Cataract formation	CRYBB1 gene; rs2289917 risk allele; Xp22.13 AD and AR inheritance	GCNT2; CHMP4B; PEX11ß; CYP51A1; SC5D, GALK1; CYP27A1; SLC2A1; AD, AR and XLR inheritance	Multiple genes regulating different lenticular activities have been delineated in the Western population [54]. More extensive Asian studies required
5	Cone-rod dystrophy and other retinal degeneration [13, 57, 86]	Damage to cone and rod cells of the retina due to defects in the phototransduction process, primary development, and differentiation of retina and photoreceptors	Difficulty in light adaptation, low vision; scotoma; nystagmus; color vision defects, progressive visual field defects	GUCY2D RPE65, LCA5, USH2A, CNGB1, FAM161A, CERKL; AR and AD inheritance	At least 30 genes have been elucidated; GUCY2D, CRX and ABCA4 account for about 50%; AR and AD inheritance	Extensive genotypic and phenotypic subtypes present as studied in the Caucasian population. Limited studies from Asia reveal similar genes
6	Aniridia [22–25, 80]	Structural abnormality in eye development Partial or complete absence of iris	Glaucoma, low vision; photophobia; cataracts; may be associated with Wilms' tumor in case of deletions affecting both the PAX6 and the WS gene	PAX6; AD inheritance	PAX6; AD inheritance and sporadic	Similar genetic profile in Asian and non-Asian populations

(continued)

Table 26.1 (continued)

S. No	Condition	Etiopathogenesis	Clinical features	Genetic aspects of reviewed Asian studies	Affected genes in other ethnic populations	Comments
7	Anophthalmia/ microphthalmia [19, 76–81, 83–85]	Structural abnormality in eye development Small eye with structural disorganization of other ocular components Significant phenotypic heterogeneity	Low to absent vision	FOXE3 (AR inheritance)	FOXE3; PAX6; RAX, BMP4; SOX2, TENM3, ALDH1A3, STRA6 (AD inheritance; mosaicism	Extensive genotypic and phenotypic subtypes identified in the Caucasian population. Limited studies from Asia reveal mainly 1 gene, as mentioned. This needs to be studied further
8	Achromatopsia [21, 87]	Channelopathy affecting primarily cones, may be progressive	Photophobia, nystagmus, decreased vision, color blindness	ATF6; AR recessive	ATF6; CNGA3; CNGB3; GNAT2; PDE6C; PDE6H; AR recessive	Extensive genotypic and phenotypic subtypes present as studied in the Caucasian population. Limited studies from Asia reveal mainly 1 gene, as mentioned. This needs to be studied further
9	Pierson syndrome (PS) [32–34]	Genetic modulation Early-onset renal, ocular and neurodevelopmental problems	Microcoria (small pupils nonresponsive to light), blindness; chronic renal failure	LAMB2; AR inheritance	LAMB2; AR inheritance	Similar genetic profile in Asian and non-Asian populations
10	Ataxia-telangiectasia-like disorder (ATLD) [12, 15, 16]	Increased fetal alpha-protein Multisystem disorder affecting the eye, nervous system, and immune system	Ataxia; apraxia, chorea, myoclonus	W210C MRE11 mutation; AR inheritance	ATM; W210C MRE11 mutations noted in Saudi families; AR inheritance	W210C MRE11 have been noted in only Saudi families in ATLD
11	Leber congenital amaurosis (LCA) [55, 73–75]	13 phenotypes described Abnormalities in retinal development comprising photoreceptors, ciliary dysfunction, phototransduction	Photophobia, nystagmus, hyperopia, nonreacting pupils, keratoconus, oculo-digital sign positive, ID, global developmental delay (GDD)	AIPL1, RPE65, GUCY2D. CRB1, RDH12, IQCB1 and SPATA7; AR inheritance	18 genes; the most common being CRB1, GUCY2D, RPE65, CEP290; AR and AD inheritance (CRX, IMPDH1)	Extensive genotypic and phenotypic subtypes identified in the Caucasian population [82]. Limited studies from Asia reveal similar genes

12	Retinitis pigmentosa (RP) [55, 88–98]	Rod-cone dystrophy due to multiple causes such as ciliopathies, structural defects, defective phototransduction	Progressive visual loss, nyctalopia, loss of peripheral vision and later also of central vision, isolated or syndromic (Bardet-Biedl; Usher; Refsum)	MERTK; USH2A; RHO (AD inheritance); CACNA1F (described previously in a Dutch population [93]; RPGR, RP2; AR inheritance	35 genes; RHO (most common for AD type); USH2A for AR type); RPGR and RP2 (XLR type)	Extensive genotypic and phenotypic subtypes identified in the Caucasian population. Extensive studies from both West and East Asia reveal similar genes
13	Congenital hereditary endothelial dystrophy (CHED) [20, 99]	Bicarbonate transporter problem [99]	Nystagmus; photophobia, corneal clouding; epiphora, deafness (Harboyan syndrome)	SLC4A11; AR inheritance	SLC4A11 (AR inheritance); AD inheritance	One gene identified for AR form in both Asian and non-Asian populations
14	Muscle-eye-brain (MEB) disease [30] Kevin Campbell Nature 2002	Abnormally glycosylated form of alpha-dystroglycan	Hypotony, myopia, glaucoma, developmental delay, ID, brain abnormalities like hydrocephalus	POMGNT1; AR inheritance	POMGNT1; AR inheritance	Rare condition; similar genetic profile in Asian and non-Asian populations
15	Conradi-Hünermann-Happle syndrome [31] chondrodysplasia punctata	Punctate Peroxisomal disorder	Skeletal dysplasia, stippled epiphyses, cataracts; ichthyosis; ID	EBP; X-linked dominant (XLD)	EBP; XLD	Similar genetic profile in Asian and non-Asian populations
16	Coats' plus disease [56] (Coat's disease plus abnormalities of the brain, bones, gastrointestinal system, and other parts of the body)	Idiopathic compound heterozygous mutations at CTC1 gene which impair the function of telomeric replication	Retinal telangiectasia, intracranial calcification, osteopenia, leukoencephalopathy, brain cysts	HES7, TEK; AR inheritance	Norrie disease gene (NDP); CTC1 gene; AR inheritance	Similar genetic profile in Asian and non-Asian populations; novel mutations in genes are different
17	CCDD-type strabismus (CFEOM, DRS) [63–67]	The KIF21A gene for a protein of the kinesin family member 21A, important for transport in developing neurons; PHOX2A gene provides instructions for a protein aiding formation and differentiation of neurons	Inability to move eyes; Tukel syndrome (associated oligodactyly)	KIF21A; AD inheritance; PHOX2A; AR inheritance found in a Saudi Arabian family	KIF21A; AD inheritance PHOX2A; AR inheritance	Similar genetic profiles; PHOX2A has been found to be affected in a Saudi Arabian family with phenotypic features of CFEOM 2 (similar to CFEOM 1 but with AR inheritance pattern)

subjects with and without pediatric cataract in a pediatric ophthalmology referral unit in Western India revealed that the rs2289917 risk allele showed a strong association with increased likelihood for pediatric cataract [8]. Another study from the state of Andhra Pradesh in India determining the prevalence of consanguinity, revealed 24.7% potential association of consanguinity with eye diseases in the rural population and 32.9% of the same in an urban population. In this study, 0.008% subjects had an ocular disease with a potentially genetic basis. Specifically, these were microcornea which showed a high association with both the maternal uncle-niece consanguinity pattern and retinitis pigmentosa, which showed an association with first-cousin marriages [9]. In a large two-step Japanese study aimed to determine the epidemiological, clinical, and genetic characteristics of congenital hypomyelinating leukodystrophies, including Pelizaeus-Merzbacher disease (PMD), the prevalence of the former was found to be 0.78 per 100,000 people, and the incidence 1.4 per 100,000 live births, while that of PMD with PLP1 mutations was estimated to be 1.45 per 100,000 male live births [10]. Nystagmus and generalized hypotony were characteristics of PMD with PLP 1 mutation [10]. These may, however, disappear with time [10]. A large review of pediatric genetic eye disease from west Asia encompassing the Arabian Peninsula and Northern Africa elaborates on homozygosity mapping as an important tool to understand genetics in the Middle East. The traditional practices of this area comprising consanguinity, endogamy, and a preference for many offsprings leads to an increased frequency of heterozygous gene mutation carriers and affected with homozygous mutations typical for autosomal recessive ocular disease, manifesting initially in children [11].

26.3 Asian Perspective

As mentioned in the previous section, Asia comprises a huge landmass with different ethnicities of populations having varied traditional models of marriage and bearing offsprings. Hence, the genetics of the populations living in West Asia becomes very different from, for example, Southeast Asia. Besides the epidemiological studies cited above, many other important ocular genetic studies relevant to this population have been conducted in different parts of the continent. As the numbers of such studies are really high, there has been specific focus that has been delegated to pediatric eye diseases [12].

Starting from West to East, the Israeli and Arabian population has a high prevalence of consanguineous marriages and consequently autosomal recessive diseases like rod-cone dystrophy. Researchers from Israel, Saudi Arabia, Turkey, and Pakistan have studied this extensively, and these have been elucidated in Table 26.1 in details.

Whole exome sequencing (WES) was used to delineate the GUCY2D gene as the cause of autosomal dominant (AD) cone and cone-rod retinal dystrophies in the Israeli population in one such large study incorporating 106 families [13]. This is similar to the Caucasian database [13]. This study of course had nothing to do with consanguinity [13]. Eighty-five novel mutations, different from the Caucasian spectrum, were found in a Chinese study incorporating screening of the ABCA4 gene for Stargardt disease, cone-rod dystrophy, and retinitis pigmentosa [14]. Ophthalmic features of an ataxia-telangiectasia-like disorder (ATLD) has been described in Saudi Arabia in 13 individuals from 3 unrelated consanguineous Saudi Arabian families harboring a W210C MRE11 mutation. None of the affected family members had any structural ocular abnormality like conjunctival telangiectasia or manifest strabismus, but convergence insufficiency, saccadic dysfunction, nystagmus with abnormalities in both the vestibular ocular reflex (VOR) and smooth pursuit were observed [12]. The molecular basis of this phenotype has not been elucidated in this article [12]. Another study predates this study, again on ten members of three unrelated Saudi families, with uncovering of the W210C MRE11 mutation in ATLD [15]. It was postulated that functional interactions among the three proteins of the

Mre11-Rad50-Nbs1 complex act as a sensor of DNA double-strand streaks, acting upstream of the ATM gene [15]. A study on nine subjects from four Saudi families confirms the presence of mutations in the APTX, SETX, and MRE11 genes in oculomotor apraxia type 1 (AOA1), ataxia with oculomotor apraxia (AOA2), and ATLD, respectively [16].

An Indian study identified a truncating mutation in the NHS gene (Nance-Horan syndrome) in a South Asian family with X-linked developmental lens opacity and microcornea. By fine mapping, the disease gene was localized to Xp22.13 [17]. In another study from South India, children with ocular coloboma without any other systemic features were recruited, their families traced, pedigrees drawn, and family members examined. It was found that in 21.4% of the cases, another family member was affected, and the risk for an affected sibling was 3.8%. Consanguineous marriage was found in 44.6% of the patients. Both autosomal dominant (AD) and autosomal recessive (AR) modes of inheritance were seen [18]. Anophthalmia and microphthalmia disorders are a heterogeneous group of disorders known to be caused by chromosomal aberrations, copy number variations, and single-gene mutations. In a study including eight consanguineous families from the Indian subcontinent, seven from Pakistan, and one from India, three novel mutations were identified in the FOXE3 gene as the underlying genetic cause, as determined by Sanger and whole exome sequencing [19]. A novel missense mutation in the SLC4A11 gene was found in two consanguineous Pakistani families affected with congenital hereditary endothelial dystrophy (CHED), first by linkage and microsatellite markers and then conformed by sequencing [20]. A mutation in the ATF6 gene was found to cause AR achromatopsia in a Pakistani consanguineous family as determined by homozygosity mapping, linkage, and exome sequencing [21].

Two recent studies from South India have thrown some light on mutational analysis and genotype-phenotype correlations in patients with sporadic and familial aniridia [22, 23]. Mutational analysis of the PAX6 gene was performed to assess associated phenotypes in patients affected with aniridia along with familial and sporadic healthy controls [22]. Thirteen different mutations were detected in the eight sporadic cases and five familial cases. Interestingly, all mutations in this study were truncations. This is quite typical, missense mutations in PAX6 being infrequent, and similar to Western database on PAX6 run from Edinburgh, UK (http://isdb.hgu.mrc.ac.uk/home.php?select_db=PAX6). Other common findings in association with these mutations seen in these patients comprised iris hypoplasia, nystagmus and foveal hypoplasia ubiquitously and cataract, glaucoma, and keratopathy in about 50% cases [22]. The other report elucidated a deletion and duplication involving 6p25, causing an aniridia-like phenotype [23]. A Korean study elucidated a splice mutation in the PAX6 gene at a splice donor sire of intron 8 in the proband (c.357_1delG) in a 1-month-old boy and his 4-year-old sister both with aniridia. The father did not have aniridia but bilateral microcornea and cataract indicating phenotypic heterogeneity and possible variable expressivity [24]. In a Chinese study, a novel mutation was found in the PAX6 gene in a 10-month child with Peters' anomaly along with congenital nystagmus, corneal leukoma, anterior synechiae, anterior polar cataract, microphthalmos, retinal agenesis, and non-dilating pupils because of posterior synechiae [25]. The PAX6 gene family and the resultant PAX proteins are called transcription factors because they control the activity and expressivity of particular genes involved in the formation of the eyes, the brain, and the spinal cord during embryonic development and help regulate expression of different genes in many structures of the eyes after birth. Its malfunctioning has been associated with conditions like aniridia, iris and fundal coloboma, microphthalmia, Peters' anomaly, Gillespie syndrome, etc. globally [26–29].

In a recent Chinese study on muscle-eye-brain (MEB) disease, a congenital muscular dystrophy characterized by muscular hypotony, myopia, structural brain abnormalities like cerebellar

cysts, brainstem flattening and kinking, as well as cognitive impairment, a novel POMGnT1 gene mutation was found [30]. A Thai study identified two novel mutations in two unrelated female children with the Conradi-Hünermann-Happle syndrome, also known as chondrodysplasia punctate type 2 (CDPX2), an X-linked dominant disorder characterized by skeletal, ocular, and skin defects [31]. CDPX2 has been shown to be due to mutations, about 58 of which have been described, in the emopamil-binding protein (EBP) gene [31]. In this study, mutation analysis was performed through PCR sequencing of the entire region of the EBP gene [31].

Pierson syndrome is a mitochondriopathy characterized by congenital nephritic syndrome and ocular anomalies like microcornea, miosis, and nystagmus. About 50 mutations have been identified in Western countries in 40 unrelated families in the LAMB2 gene, coding laminin β2, as being disease-causing. The first Japanese and the first Chinese (Asian) case reports described mutations in the same LAMB2 gene [32, 33]. Since then, variable phenotypes of this condition have been described in additional patients from the Asian population [34].

Nystagmus comprises repetitive, involuntary eye movements, which are often associated with poor vision. Besides acquired conditions such as retinopathy of prematurity, also conditions with inborn defects in the retina such as congenital achromatopsia, blue cone monochromacy, congenital rod-cone dystrophy, Leber congenital amaurosis LCA, and various forms of congenital stationary night blindness are often associated with typical sensory defect nystagmus or abnormal searching eye movements. X-linked congenital nystagmus was linked to mutations in the FRMD7 gene located in the FERM domain of the X chromosome in a large linkage study in the UK [35]. It is interesting to note that there were Chinese studies preceding this landmark study that had isolated a genetic locus on the X chromosome that was later found to conform to the FRMD7 gene domain [36, 37]. Subsequent studies in the Chinese population with X-linked congenital nystagmus have identified mutations in the FRMD7 gene thus confirming the location in the Asian population also [38–43]. In one large Indian family comprising 71 members over 11 generations, affected with familial X-linked congenital nystagmus, a novel missense mutation c. A917G in the FRMD7 gene were found [44]. The mutation, located at a highly conserved residue within the FERM-adjacent domain in affected members of the family, was detected in hemizygous males and in homozygous and heterozygous states in affected female members of the family. The disease was fully penetrant in all males with the mutation ($n = 9$). All homozygous affected females ($n = 8$) demonstrated complete expression of the phenotype. Heterozygous affected females ($n = 3$) and obligate female carriers without disease ($n = 5$) demonstrated incomplete or no demonstration of the phenotype, which could be attributed to an incompletely penetrant gene, consistent with a previous report in the Western population [45]. The report elucidates that the clinical significance of hemi-/homozygous and heterozygous mutations is not very clear. They report that a missense mutation, R229G, in the FRMD7 gene in heterozygous and homozygous condition in members of a large family with X-linked congenital nystagmus was found without any phenotypic variation [46]. In the current study, elucidating the Indian family, the mentioned mutation was not detected in unaffected members of the family or in 100 unrelated control subjects [44].

X-linked ocular albinism (XLOA) has been studied in a Chinese population comprising six families with XLOA. Mutations in GPR143, one known and five novel, were identified in all six families [47]. Nystagmus, poor visual acuity, and foveal hypoplasia were present in all affected patients. However, iris and fundus hypopigmentation were minimal [47]. This may be in part due to relatively increased pigmentation in the Asian population, often making the differential diagnosis between X-linked nystagmus and nystagmus due to ocular albinism particularly difficult [48]. One Chinese study reports GPR143 mutations in five Chinese families initially diagnosed as having X-linked congenital nystagmus [49]. Twenty-seven patients from

24 Indian families were screened for causal variants for different forms of autosomal recessive albinism by PCR sequencing. Tested were the following genes: TYR, OCA2, TYRP1, SLC45A2, SLC24A5, and SILVA; novel insertion was detected in SLC24A5 in a patient with extreme hypopigmentation. Further, five TYR and three OCA2 mutations were found [50].

As mentioned before, Pelizaeus-Merzbacher disease (PMD) is a rare X-linked recessive demyelinating disorder associated with nystagmus, impaired motor development, ataxia, and spasticity. RT-PCR, genetic linkage, and SRY sequence analysis were performed on two large Han Chinese families and disclosed disease-causing mutations. In both families, the probands had been diagnosed with cerebral palsy [51]. The phenotypic variation of PMD spans from the most severe forms of disease-causing neonatal morbidity to milder symptoms compatible with life, like nystagmus. A novel proteolipid protein (PLP) gene mutation was described in a Japanese patient with milder symptoms of PMD [52].

Clinical and molecular analysis of children with central pulverulent cataract from Saudi Arabia identified as candidate gene CRYBB1 in one consanguineous family. The cataract is usually not visually significant and compatible with good visual acuity [53]. Many genes have been described in the Western population as being causative of congenital cataract. They are referenced in Table 26.1 and are not repeated here to maintain brevity [54].

Both Leber congenital amaurosis (LCA) and retinitis pigmentosa (RP) are retinal degenerative disorders with a genetic basis, mostly autosomal recessive, but also autosomal dominant and X-linked (RP). Homozygosity mapping was used as a tool to localize relevant known and novel disease gene mutations in 11 consanguineous LCA and one AR RP family from Southern India, to determine the prevalence of mutations in known genes, and also to possibly detect novel loci. Gene mutation identification was done using the Affymetrix 250 K GeneChip in the 11 LCA families. Ten mutations, six of them novel, were found in the following candidate genes: *AIPL1*, *RPE65*, *GUCY2D*, *CRB1*, *RDH12*, *IQCB1*, and *SPATA7*. In the single family with ARRP, a novel nonsense mutation was found in MERTK [55]. WES was effective in uncovering a rare homozygous missense variation in the TEK gene in a child with Coats' disease [56]. Retinal blinding disorders with a worldwide prevalence of 1 in 2000 represent a significant impact on the quality of life. It is important to note that mutations in genes expressed in RPE cells, photoreceptors, or bipolar cells all can result in varying forms of degenerative or stationary retinal disorders, as the presence of the encoded proteins is crucial for normal function, maintenance, and synaptic interaction. The degree of damage caused by different mutations depends upon the type of mutation within the gene, resulting in either total absence or the presence of a nonfunctional or potentially toxic protein [57].

Primary concomitant strabismus (PCS) like intermittent exodeviations and accommodative esodeviations also demonstrate hereditary patterns [1]. The majority of studies have proposed polygenic inheritance with varying interplay of genetic and environmental factors, as simple Mendelian models cannot explain the patterns. Genome-wide searches and linkage analysis have been used to locate genes related to strabismus. The most significant finding so far was localizing the recessive $STBMS_1$ locus on chromosome 7p22.1 [1]. Other susceptibility loci have been localized on chromosomes 4, 6, 9, 12, and 19. [1] Mutations of the kinesin KIF21A and RYR1 receptors have been associated with monogenetic forms of strabismus, comprising the congenital cranial dysinnervation syndromes (CCDDs) like congenital fibrosis of extraocular muscles (CFEOM), Möbius syndrome, and Duane's retraction syndrome (DRS), which demonstrate clear monogenic inheritance patterns [58, 59]. The transcription factors SALL4 and HOXA1 were identified as genes mutated in patients with DRS associated with radial abnormalities and cognitive defects, respectively [60]. Isolated DRS can segregate as autosomal dominant and was mapped to the DURS2 locus [60]. It was reported that recessive COL25A1 mutations caused congenital ptosis and exotropic

DRS with synergistic divergence in two Saudi Arabian families [61]. Further research on large groups of affected individuals and families is required to identify causative genes in these relatively common ocular conditions. Such efforts may help understand the phenotypic variability in strabismus patients and may also help identify individuals at risk and spare them the significant morbidity associated with this common disorder.

To date, Asian data does not contribute in a substantial way to the genetics of strabismus. A recent study describing the clinical, radiological, and genetic features of the blepharophimosis syndrome in 33 Indian patients revealed a high incidence of refractive errors (94%), amblyopia (60%), and strabismus (40%) in this population. Chromosomal anomalies were present in 8 of these 33 subjects, all on chromosome 3, and cytogenetic and molecular analysis of the FOXL2 gene revealed a novel mutation, i.e., a heterozygous substitution at c1635. This study incorporated strabismus as a clinical parameter but no analysis that compared the strabismic and non-strabismic subjects for genetics [62]. An older study evaluated CFEOM in an Indian family by linkage analysis. Microsatellite markers were used for linkage on 2 known dominant CFEOM loci on chromosomes 12 and 16 and were found to be consistent with existing literature [63]. In a more recent study in a Chinese family, maternal germline mosaicism of the KIF21A mutation was postulated to result in complex phenotypes of CFEOM type 1 in view of the fact that a heterozygous hotspot mutation was found in all three affected siblings but was absent in both parents and haplotype analysis of the diseased locus showed likely maternal inheritance. Again, as in the previous Indian study, microsatellite markers were used across the KIF21A locus [64]. Another Chinese study found the presence of KIF21A mutations in two Chinese families, thus confirming that mutations in KIF21A are a cause of CFEOM in different ethnic populations [65].

A Saudi Arabian study postulated absence of KIF21A in consanguineous families with CFEOM, opening up the possibility of also recessive inheritance of this condition [66]. This result is similar to a previous study on mutational analysis of the PHOX2A/ARIX gene in an Iranian family with CFEOM which was compatible with AR inheritance in these patients [67].

In a linkage study by a Saudi Arabian group on a consanguineous family, with members exhibiting different phenotypic forms of childhood strabismus, a single disease locus on chromosome 16, 6 MB in size and comprising 80 genes was localized, postulating that different phenotypic forms of strabismus, i.e., infantile esotropia, esotropic DRS, esotropia and anisometropia, and monocular elevation deficiency (MED), all share the same genotype [68]. Again, in another Saudi Arabian study by the same group, phenotyping and linkage analysis was performed in a consanguineous nuclear family comprising 12 children and both parents. Three children had non-syndromic large-angle infantile esotropia, the fourth child had left esotropic DRS, the fifth child had esotropia with retinopathy of prematurity, and the sixth had keratoconus. The last two children were excluded from the analysis. The remaining six children and the parents were orthotropic. It was concluded that non-syndromic infantile esotropia could be related to susceptibility loci on chromosomes 3 and 6, which persisted even when the data of the child with DRS were excluded from the linkage analysis [69]. Proteomic analysis of infantile esotropia phenotypes in monozygotic twins with discordance of congenital esotropic phenotypes by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TPF-MS) revealed four differentially expressed proteins similar to the glucagon precursor, pituitary adenylate cyclase-activating polypeptide (PACAP), camp-dependent protein kinase inhibitor α, and anti-metastasis antigen, respectively. It was postulated that these differentially expressed proteins were mainly downregulated in strabismus patients versus normals and could thus be involved in the occurrence and development of infantile esotropia in these patients [70].

Thus, while the CCDDs and more particularly the CFEOMs have been widely studied in both

East and West Asia, there are no studies on the genetics of other forms of strabismus.

As a corollary, as mentioned previously, the Asian pediatric ocular genetic disease profile is also significantly variable in different regions of Asia [4–14, 16–25, 30–32, 34, 36–42, 44, 47, 50–53, 55, 56, 63–75]. Some of the localized mutations and affected genes are similar to the Caucasian profile and some show variations [1, 15, 35, 54, 76–85]. In this context, it is interesting to note that genetic profiling of ATLD has been performed only in Saudi Arabian families with the uncovering of the W210C MRE11 gene mutations [12, 15, 16]. Genetic analysis of many known syndromic and non-syndromic conditions is a branch, which is continuously getting updated and adding to human knowledge.

26.4 Etiology, Pathology, Clinical Features, Genetic Aspects

The genotype-phenotype profiles of some common pediatric ocular disorders and strabismus in Asia reported to date are summarized in Table 26.1.

Table 26.1 summarizes many of the Asian genetic studies conducted in different parts of West Middle, South, and East Asia, among different ethnic groups. Most of the studies were set to screen candidate genes already identified in the Western population. Many such studies found novel mutations in known genes. Newer technologies comprising WES and whole-genome sequencing (WGS) have resulted in unmasking many more loci and genes with many novel mutations. However, the causative nature of many of these novel mutations, else from being mere polymorphisms, remains to be functionally evaluated.

It is interesting to note that most of the mutated gene domains were found to be the same in the Asian population as observed in most other ethnic populations. Consanguinity among the South Indian population and the West Asian populations could unmask many genetic diseases that would otherwise go unclassified. With the advent of WES and WGS, more novel mutations on known and novel genes are likely to be isolated and should greatly add to global literature from different parts of the world, including Asia.

26.5 Molecular Biology and Laboratory

The molecular biology profile and laboratory tests to elucidate valid genetic interpretations have been detailed in different chapters of this book and will therefore not be repeated here. Molecular biology is specifically a synthesis of genetics and biochemistry. It is interesting to observe the metamorphosis of genetics from being a semi-epidemiological branch with pedigree analysis and genotype-phenotype correlations as its main armamentarium transform into a highly sophisticated biotechnological science incorporating experimental and bioinformatics tools requiring deep intrinsic knowledge of both the biological and the mathematical sciences. In the scientific studies quoted in the text above, many genetic tools including homozygosity mapping (efficient strategy for mapping known and novel loci in recessive conditions, especially in consanguinity), single-nucleotide polymorphism (SNP) array, linkage, PCR sequencing, WES, WGS, and genome-wide association study (GWAS) were used. However, genotype-phenotype correlations and a good pedigree analysis are still the basis on which these tools are used for further detailed interpretations, especially functional in vitro assays which are increasingly becoming more important to identify whether a mutation, novel, or known is truly associated with the causation of the disease or is just a normal polymorphism. In vivo studies require extensive work on figurative animal models, which may not be a feasible option for many clinical science setups.

26.6 Summary

Pediatric eye diseases including strabismus comprise a wide range of hereditary conditions. This chapter provides an overview of the major

studies done in the Asian continent to date. The data was compared to that found in the Caucasian population. A typical finding was that while the affected genes remained similar in different ethnic populations, novel mutations were frequent and different, thus extending the mutation spectrum for the concerned clinical condition. The next step would be functional validation of these mutations, not only in the Asian population but also in the Caucasian populations in Europe and America. Hotspots of extensive genetic studies in Asia have been in Saudi Arabia, Pakistan, India, China and Japan. The genetic diseases likely correlate with social customs in these ethnic populations, especially AR inheritance pattern related to consanguinity which is very common in the Arabic Peninsula, Pakistan, and South India and which would thus be expected to result in higher prevalences of these conditions than in other parts of the world.

Compliance with Ethical Requirements Dr. Zia Chaudhuri and Dr. Birgit Lorenz declare that they have no conflict of interests.

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Keratoconus: Globally and in the Middle East (Epidemiology, Genetics, and Future Research)

27

Ahmed Mousa, Altaf Kondkar, and Khaled K. Abu Amero

Abstract

Keratoconus is a non-inflammatory thinning and anterior protrusion of the cornea that results in steeping and distortion of the cornea, altered refractive powers and altered visual acuity. Therefore, KC is one of the indirect causes of low vision and blindness (as it leads to refractive errors). The prevalence of KC is relatively high worldwide, with a special increase in prevalence and incidence among the Asian communities. However, this prevalence suffers a considerably high range of variation. It can be recognized that such prevalence is increasing with moving geographically towards the east. Epidemiologically, there are a number of potentially associated risk factors for KC. These factors may include: genetic profile, consanguinity, geographic latitude, exposure to direct light (Especially UV) and personal behaviors (such as eye rubbing). Genetically, there is a number of associated genes and SNPs that were proved to be associated with KC. Various studies have indicated that it is quite possible that there are more than one gene contributing to the development of KC. To date, keratoconus was found to be associated with around 17 different genomic loci. Moreover, research continues for detecting more associated genes and SNPs with KC. Meanwhile, management of KC is now available and accessible. Recently, KC treatment modalities have drastically improved. Such treatment modalities would imply Corrective Glasses and contact lenses, implant of Intraocular lenses, Cross linking procedure, Intra-corneal Ring Segment, Kertatoplastic surgeries and Photrefractive Keratectomy. However, some of the cases either may present

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late to eye care services or even never sought such services, while there is no early detection and referral mechanisms in place yet.

Keywords
Keratoconus • Ophthalmic Genetics

27.1 Terminology

The word "*keratoconus*" (KC) is following a combination of two words of Greek origin, those are KERATO = cornea and KONOS = cone, which together formulate the meaning "*a cone-like protrusion of the cornea*."

27.2 Definition

Keratoconus (KC) is a noninflammatory thinning and anterior protrusion of the cornea that results in steeping and distortion of the cornea, altered refractive powers, and altered visual acuity. In more advanced cases, corneal scarring from corneal edema and decompensation further reduces visual acuity [1, 2].

27.3 Etiology

Although keratoconus affects all different ethnicities, there is a documented evidence that it affects certain ethnicities more than others. This fact is supported by the relatively high variation in the assessed prevalence among different ethnic groups [3, 4]. Globally, the prevalence of keratoconus is highly variable across different regions of the world [5]. This high variation is reported as a range between 0.3 per 100,000 and 15,500 per 100,000 inhabitants in Russia and Yemen, respectively. However, such variation is considerably high in terms of epidemiological measures. Meanwhile, the reported incidence of KC from different regions, which ranges from 3.3 to 25 per 100,000 inhabitants, may also reflect a wide range of variation from the epidemiologic point of view [6].

The potential rationale behind this relatively high variation of KC prevalence and incidence is the impact of some environmental, ethnic, and sampling factors. However, other associated factors such as gender and family history of disease may play a major role in such variation [5]. Moreover, the diagnostic indications as well as the assessment method may also contribute in reaching up to a confirmed diagnosis of KC.

Generally speaking, the actual etiology of KC is yet unclear. What is understood to date is that it is multifactorial in nature and its development and progression pathway is interactive across different factors such as genetic profile, personal attitudes, geographic locations, and many other aspects.

27.4 Pathology

Pathologically, KC is a result of basal epithelial cells degeneration that leads to distortion of the basement membrane. Such disruption yields an abnormal growth of the epithelial cells posterior to the bowman's layer which by its turn and under some prognostic factors ends up with the abnormal shape of cornea identified as KC. This process is consequently followed by pathogenic degeneration of the basement membrane. Progressive degeneration usually follows at a variable rate dependent on various factors as mentioned earlier [7].

27.5 Signs and Symptoms

The clinical symptoms of KC start either in youth or early adolescence stages with a bilateral presentation in the majority of cases with one eye more advanced than the other. KC has some early signs that would include blurred vision and

frequent need to update distant glasses, if any, till reporting that vision cannot be corrected with glasses anymore. Some other symptoms may include increased sensitivity to light, difficultly to see well at night, recognizing a halo around lights especially at night, eye strain, irritation, allergy, desire for rubbing, and pain. KC, especially in the early and mild stages, is difficult to diagnose, with the fact that most of its symptoms can be easily attributed to other ocular conditions. Therefore, detection of KC may need examination by an anterior segment or cornea subspecialty consultant as well as inspection of the cornea at a microscopic level using a slit lamp. Later on, the diagnosed thinning and protrusion of the central part of the cornea would be more explicit. This would be mainly due to further degeneration of the anterior corneal layers where the primary lesions are usually found in the basic cells of the epithelium [8].

27.6 Diagnosis

Since KC was first introduced, several improvements in the diagnostic and detection methods took place. The pathophysiology of KC clarifies that the disease is multifactorial in nature. Therefore, the continuous efforts to understand KC has revealed that several molecular changes take place in a cascade of events. This fact has ultimately changed the understanding of KC from being considered as a mysterious disease to documenting a series of prognostic mechanisms. Thus, recently, pathophysiologic pathways of KC are widely classified in terms of alteration of stroma composition, imbalance of pro-inflammatory and anti-inflammatory molecules, and imbalance of the enzymes that cause extracellular matrix degradation and their corresponding inhibitors, oxidative stress, and cellular hypersensitivity. However, although these events occur in a cascade of events module, yet, there is no clear idea about the inter-factorial correlation, interaction and impact of some of them on one another [9].

The classic way of KC assessment included external eye examination using anterior segment expert opinion plus utilization of same widely used scales such as the Amsler-Krumeich scale (which is still being in use) to classify KC. However, the availability of electronic and molecularized methods of assessment such as the elevation-based slit scanning and topographic mapping in addition to the Schein plug cornea imaging using Orbscan and Pentacam has dramatically increased and is nowadays considered as an essential tool for differential diagnosis and categorization of KC [10].

Nowadays, advanced diagnostic method has enabled more accurate detection of KC status, better ruling in positive cases, better grading methods, and even more enhanced methods for detection and follow-up of prognosis. Research and development have also yielded a number of clinical ideas that can be easily used to diagnose and categorize the stage of KC and its level of severity. One of the very helpful tools in this regard is the computerized corneal topography and tomography assessment instruments. These methods have enabled fine screening of the central corneal steeping, asymmetry of optical power, and asymmetry of the interior corneal steeping as well. One of the great advantages of using these newly developed techniques and the corresponding indices is that its capability to detect some of the subclinical cases and rule them in. As the association between KC and genetic makeup is well documented in the literature, there is always a need – from the epidemiologic point of view – to screen the "at high-risk group" and calculate the risk of encountering the disease among them [11].

27.7 Prevalence and Incidence

Many international studies have tried to assess the prevalence, incidence, and potential risk factors for KC. However, the published reports show a wide range of variation in terms of both prevalence and risk factors for KC. Overall, there is a more or less agreement that the prevalence of KC is highly affected by a number of controlling factors, the most important of which are the methods of assessment and the diagnosis –

criteria. This fact may hinder the possibility to compare findings of different international reports. For example, publications from Russia, India, Iran, the USA, Columbia, Pakistan, Bangladesh, Yemen, the UK, and other countries show a high variation in the reported KC prevalence. It is quite astonishing that this variation is ranging between quite high and quite low figures such as Russia, $0.3/10^5$; India, $2300/10^5$; Iran, $2500/10^5$; the USA, $54.5/10^5$ and $25{,}500/10^5$; Columbia, $3900/10^5$; and Yemen, $15{,}500/10^5$ and $9400/10^5$ (R1). This remarkably high variation in the assessed prevalence is mainly due to – however not limited to – the variation in both assessment method and criteria as mentioned. For example, the Indian study indicates that a KC case is clinically confirmed if the corneal refractive error is $\geq$ 48 diopters. Meanwhile, the Iranian study uses a pure clinical assessment method via retinoscopy, slit lamb examination, and corneal topography as a method of assessment of the KC. Nevertheless, it is quite clear that many other geographic, methodological, and study design-associated factors are not less important than the mentioned factors. Such factors can be further discussed as follows: First, study design wise, it should be clear that the majority of the published literature is reporting hospital-based studies. Therefore, it is highly susceptible to "selection bias" as it withdraws its sample either from cases that present to eye care hospitals for conduct of refractive error surgeries or from those who present to corneal clinics in general, the matter that would definitely implies a bias that lead to overestimation of the prevalence. Second, regardless of the assessment method, tool, and criteria, almost all of the used methods would either totally or partially miss the mild and subclinical cases, which can be further identified as "those who did not reach up to the clinical diagnosis threshold." Those two additional factors may be also considered as limitations for proper capturing of KC cases and, hence, accurate estimation of the KC prevalence [12].

Nevertheless, it was found that there is an explicit effect of the geographic location on the prevalence, incidence, and severity of KC. Carefully looking at the available literature, one can easily distinguish the fact that as you go geographically to the eastern part of the earth, the prevalence of KC is more or less increasing. This proven fact is further assured by reporting the highest prevalence among the Asian population.

On the other hand, risk factors for KC were also a matter of discussion among the scientific community in the last three decades. Findings from different studies refer to potential association between KC prevalence, gender (more likely to occur in males than females) and race (Asians are more affected than Caucasian whereas studies from Pakistan and Bangladesh demonstrate that the prevalence there is 4.4–7.5 times higher than the Caucasian prevalence) [13, 14]. Moreover, in 2000, A. R. Pearson et al. discussed the issue of race solely in a specific report from the UK. The study which retrospectively reviewed a large number of medical records of a tertiary referral hospital over a 10-year period showed that the prevalence of KC among the Asian reviewers was $229/10^5$ compared to $57/10^5$ among the Caucasian group. Despite that the percentage of the Asian reviewers was 11% and the Caucasians' was 87%, the prevalence among the Asian group was almost fourfolds greater than the Asians' prevalence. The study has also revealed that not only the prevalence is higher among the Asians but also the onset of disease is presenting earlier in age among them [13]. The issue of early presentation among Asians was not first introduced by this paper as many other studies have pointed out and discussed the same phenomenon. The detected age interval at which the Asian population usually get first diagnosed with KC is estimated at 20–30 years [4].

Additionally, some other studies mentioned that exposure to light, specifically ultraviolet rays, personal attitudes and behaviors (such as rubbing the eye), consanguinity, and geographic latitude, as well as the genetic profile (which will be further discussed in details later in this chapter, being one of the very crucially attributed risk factors for KC), are of the effective risk factors for encountering KC. Nonetheless, it is worth saying that issues such as age at presentation,

gender bias, race, exposure, consanguinity, and genotype–phenotype association would need conduct of large community-based rather than hospital-based studies to provide a concrete evidence on such proposed hypotheses [5].

27.8 Association with Low Vision and Blindness

Keratoconus by itself is not a direct cause of low vision and blindness. However, the progression of KC would lead to thinning of the cornea and hence gradually develop a kind of corneal bulge. It is anticipated that during such progress, the cornea gradually loses its dome like shape and starts to incorrectly pass the light into the lens and retina. Moreover, further progression would lead to distortion of the lens function via the effect of swelling and scarring. Hence, all these indirect effects are highly associated with the light perception and accordingly with development of different types of refractive errors. Therefore, KC can be an indirect cause of blindness either due to direct formation of a refractive error or a corneal scaring. Both causes are of the major avoidable causes of low vision and blindness worldwide. As refractive errors by itself contribute 43% of the total visual impairment causes. This dilemma is even worse among children as estimated 12/19 (63.2%) million children with visual impairment are mainly caused by refractive errors [15].

Consequently, KC is one of the major causes of refractive errors among adults and children. On the other hand, globally, uncorrected refractive errors are responsible for getting 153 million individuals visually impaired (145 million with low vision and 8 million blind subjects) [16]. Although the exact contribution of KC in such huge burden is yet unknown, the corresponding economic loss is relatively high in terms of treatments and the loss in labor force and productive years. Furthermore, the literature in this area highlights the fact that even this high magnitude suffer a sort of "underestimation bias" as we only register the needy subjects or those who seek service because they reached an urging level of decreased vision and/or other visual functioning problems. In other words, we only see those who have visual acuity of $<$ 6/60 and 3/60 (as per the WHO definitions for legal low vision and blindness). This means that we are not counting those who are "normal" by WHO definition; accordingly, if we include anyone with decreased vision (VA $<$6/18), the magnitude would drastically increase from 153 to 500 million (more than triple folds). Moreover, published literature from Asia and Africa and other developing countries demonstrate that around 94% of those suffering from refractive error problems have not any corrective tools including even simple glasses [17, 18].

A recently published study from Italy, that attempted to assess the prevalence of blindness from KC among a long cohort of patients, showed 77/6399 (1.2%) were legally blind. However, after exclusion of all cases that had any ocular comorbidity, only 23 eyes of 17/6399 (0.3%) patients were blind and attributed to KC. Meanwhile, further in-depth analysis of the direct causes showed that the most associated causes were trauma (incidence of rupture globe) followed by failed corrective surgery and decline for surgery. Moreover, it is worth mentioning that there is also a considerable number of patients that remain blind due to unfeasibility of the proposed surgical intervention [19].

27.9 Associated Genes

27.9.1 Keratoconus-Associated Genotype

Reports of monozygotic twins, familial clustering, and increased risk of keratoconus observed in first-degree relatives strongly support that keratoconus is heritable [4, 20, 21]. Identification of causative genetic biomarkers is therefore believed to be a valuable tool in early diagnosis and future management of the disease. However, despite the familial forms of keratoconus, a majority of the disease are found to be sporadic and polygenic in nature making it difficult to identify the causative gene(s). Despite these challenges, linkage studies have reported at least 17 different genomic loci that have been associated with keratoconus as

reviewed elsewhere [22, 23]. However, only few loci among these have been replicated independently [24] and potentially pathogenic mutations in two genes including a micro-RNA (miRNA) gene *MIR184* (OMIM 613146) and *DOCK9* (*dedicator of cytokinesis 9*; OMIM 607325) have been found to be associated with keratoconus in large keratoconus family of Northern Irish and Ecuadorian origin, respectively [25, 26]. miRNAs are one of the epigenetic regulators controlling more than 60% of protein-coding genes and are known to affect several disease phenotypes [27]. *MIR184* is abundantly expressed in the cornea and lens epithelia and mutation(s) in the seed region of *MIR184* is likely to affect its function and modulate the disease outcome via deregulation of inositol polyphosphate phosphatase-like 1 gene (INPPL1) expression [28]. However, a subsequent study reported that *MIR184* variants account for only 0.25% (2/780) keratoconus cases analyzed [29]. In addition, a recent study by Abu-Amero and colleagues failed to identify any mutations in the seed region of *MIR184* in 134 Saudi patients with keratoconus indicating that variants in *MIR184* gene may be a rare cause of keratoconus and do not account for isolated or sporadic cases of the disease [30]. Similarly, in Polish patients with sporadic keratoconus variants in *DOCK9* were present only in small number of studied patients indicating that this gene may also have minor roles to play in keratoconus pathogenesis [31]. *DOCK9* is expressed in the cornea and specifically activates the cell cycle regulator, CDC42, a G-protein. Although the exact mechanism by which *DOCK9* mutation may cause keratoconus remains unexplained, a recent study by Karolak and colleagues demonstrated that a c.2262A>C variation in exon 20 of *DOCK9* leads to exon skipping. This resulted in disruption of the functional domain of DOCK9 which may affect its role as a CDC42 activator [32]. As of yet, there are no published reports of association of *DOCK9* variation(s) in the middle-east population.

Considering the complex etiology of the disease, many investigators have also employed candidate gene approach targeting potential genes involved in craniofacial and ocular development, extracellular matrix, collagens, apoptosis, and oxidative stress-related pathways to identify genetic markers associated with keratoconus, particularly in sporadic cases. Of the number of candidate genes studied so far, *visual system homeobox 1* (*VSX1*; OMIM 605020) has been most widely studied in different population. Mutations in *VSX1* genes were implicated in posterior polymorphous corneal dystrophy (PPCD) [33]. Since then, numerous studies have investigated this association in keratoconus [34–37]. *VSX1* is a member of the paired-like homeodomain transcription factor family that plays a role in craniofacial and ocular development. *VSX1* is expressed by keratocytes in injured corneas and is associated with fibroblastic transformation suggesting its role in wound healing and corneal diseases [38]. It seems that VSX1 plays a role in several pathways of keratoconus pathogenesis, but further studies are needed to validate it. However, results of genetic variations in this gene have been contradictory, and this includes a study from our center in Saudi cohorts that failed to detect any association [39–41]. Data from various genetic association studies among different population suggests that variations in *VSX1* account for only 2–3% of keratoconus cases and may not have a major role in the molecular etiology of keratoconus reflecting the polygenic nature of this disease.

Since oxidative stress plays an important role in the pathogenesis of keratoconus [42], role of genetic variants in *superoxide dismutase 1* (*SOD1*) gene localized on chromosome 21 and mitochondrial genome has also been investigated. Although an intronic deletion in *SOD1* was found to segregate in two small families with keratoconus [43], no pathogenic mutations in *SOD1* were identified in the Saudi cohorts [39, 44], and it seems that variations in this gene may account for less than 1% of keratoconus cases. Keratoconus corneas exhibit more mitochondrial DNA (mtDNA) damage than normal corneas [45]. Many variations in the mtDNA including two novel frameshift mutations in *ND1-6* mitochondrial complex I have been reported in keratoconus patients negative for *VSX1* mutations [46]. Similarly, Abu-Amero and colleagues had recently shown

that mtDNA mutations and mtDNA haplogroup H and R were significantly associated with risk of keratoconus in patients from Saudi Arabia [47, 48]. In addition, mtDNA copy number was found to be significantly increased in patients with keratoconus as compared to the normal control subjects [49, 50]. The findings of mtDNA abnormalities in keratoconus have been consistent at least in the Saudi population. However, it is essential that future studies in large cohorts of multiple ethnic groups are performed to establish the potential role of oxidative stress in keratoconus and the relevance of mitochondrial genes which at least in part may provide insights into the pathophysiological mechanisms of this genetically complex disease.

With advances in high-throughput genomic technologies, genome-wide association studies (GWAS) have also been performed in keratoconus. Using GWAS, single nucleotide polymorphism (SNP) rs3735520 at the *hepatocyte growth factor* (*HGF;* OMIM 142409) locus was found to be associated with keratoconus in cohorts from Australia, the USA, and Northern Ireland [51]. HGF promoter shows the presence of binding sites for interleukin (IL)-6, thus it appears that HGF may influence keratoconus via the inflammatory pathways. Similarly, a SNP rs4954218 located near *Rab3 GTPase-activating protein subunit 1 (RAB3GAP1*; OMIM 602536) was identified as a potential susceptibility locus for keratoconus in the US Caucasian cohorts [52]. RAB3GAP is involved in exocytosis but its precise role in keratoconus eye is not known. An association of two SNPs, rs2956540 and rs10519694, in *lysl oxidase* (*LOX*; OMIM 153455) gene with keratoconus has been reported in both family-based and case-control studies [53]. Furthermore, SNPs rs3735520 and rs2956540 in the *HGF* and *LOX* genes, respectively, have been recently suggested to serve as genetic risk markers in keratoconus patients of European descent [54]. LOX, a critical enzyme of the biogenesis of connective tissue, is involved in corneal collagen and elastin cross-linking and believed to have therapeutic implications [55]. Identification of quantitative trait loci is also an important genetic tool that can, at least in part, provide insights into the mechanism (s) involved in the progression of keratoconus. In an attempt to test for association with intermediate phenotypes and identify genes/loci associated with quantitative traits, 16 new locus were found to be significantly associated with central corneal thickness (CCT) and keratoconus in a large European and Asian population-based sample [56].

Several other candidate genes including *ZEB1*, *TGFB1*, *FLG*, *ZNF469*, interleukin (*IL1A*, *IL1B*), and collagen (*CO4A3*/*COL4A4*) have also been reported to be associated with keratoconus [57–62]; however, their role in the pathogenesis of keratoconus require further investigations. High-density SNP arrays and whole genome/exome sequencing technologies have now made it possible to identify causal genetic variants in both familial and sporadic forms of keratoconus at a much rapid pace, and more discoveries are expected in the coming years. Until then, however, the molecular pathogenesis of corneal thinning and degradation of extracellular matrix in keratoconus remains heterogeneous.

27.10 Management

During the last two decades, with the extensive increase in research in the field of KC management which is also supported by the logarithmic advances in both diagnostic and intervention medical industry as well as the innovative enhancement in surgical interventions and medical industry in general, KC treatment modalities have drastically improved. Therefore, within the context of the literature, there is a considerable agreement that the decision of intervention should be based on the possibility of conducting refraction. Thus, if refraction is available, further categorization will be necessary to understand whether such refraction is stable or unstable. Whereas, in case of unavailability of refraction, further sub-categorization based on the presence or absence of scar will be required. Two more classes of indices would indicate selection of the most appropriate method of intervention; those are corneal thickness and age intervals [10].

27.11 Corrective Glasses and Contact Lenses

The first common line of treatment in KC is applying corrective glasses as an immediate intervention – if appropriate; however, shortly, the ophthalmologist would prefer to prescribe contact lenses, which is usually of the soft type. The advantage of using such contact lenses is that it is quick, easy, and comfortable plus being financially convenient. Nevertheless, once progression starts to take place, there will be a need for quite specific contact lenses use where many of these advantages may get vanished [63].

27.12 Intraocular Lenses

Similarly, the option of inserting an intraocular lens is also quite available. Several studies provided evidence that this intervention may be highly effective with minimal side effects. In which, either anterior or posterior phakic intraocular lens is inserted which provides effective correction of vision with the advantage of relieving the contribution of patients in terms of utilization and care [64].

27.13 Cross-Linking

Collagen cross-linking is another interventional option with a critical advantage than using a corrective aid. The previously mentioned methods may provide temporary correction; however, it does not deal with the progression at all. In contrary, collagen cross-linking provides covalent bonding between the collagen molecules where it works as a scaffold that consequently works against the progression of KC in addition to reducing its undesired impact on vision. However, the effect of corneal stiffness is usually focused in the anterior thickness of the cornea rather than the rest of corneal areas [65]. Moreover, several studies provided evidence that cross-linking has not only treated the effect of KC and increased the stability of the cornea but also slowed down the progression of KC in the affected corneas [66].

27.13.1 Intracorneal Ring Segment

This technique implies inserting a ring made of inert material into the corneal stroma. The application of such ring is usually done with a mechanical creation of a specific channel (that can be also done using femtosecond laser. This procedure leads to shortening of the corneal arc length where the central cornea gets flattened, and hence the vision improves due to the consequent correction in refraction. This procedure is highly recommended in severe KC cases, while in mild cases, it may lead to minor deterioration in vision. Additionally, there are a number of potential side effects that may include infection, haze, and neovascularization ([1], [67]).

27.13.2 Keratoplasty

Keratoplasty is another option for KC management which can be further categorized into several numbers of procedures that may include "penetrating KP," "deep anterior lamellar KP," "conductive KP," and other types. Generally speaking, penetrating KP is usually indicated when there is either intolerance of the corrective contact lenses or when the vision is not getting improved anymore. In the deep anterior lamellar (DALK), all the area is removed till the Descemet's membrane. This procedure was introduced when the sophisticated investigative tools became already available. The major advantage of such technique is that it is considered as more or less an extraocular technique. On the other hand, its disadvantages are the long duration of surgery, developing myopia, and the potential risk of rejection [68]. On the other hand, conductive KP is another alternative technique that is highly recommended with increased hyperopia or presbyopia. In this technique, the cornea is being reshaped using radiofrequency energy. Although some studies have reported acceptable success rate of such procedure, it is

still considered as temporary rather than permanent method of KC correction [69].

27.13.3 Photorefractive Keratectomy

Photorefractive keratectomy is another potential option for KC correction. There is an increasing evidence that this procedure would effectively halt the progression of KC and interrupt the pathologic process of KC progression. Findings from a recent evaluative study reported favorable outcomes of this procedure, although it is still in need for conducting more in-depth investigations with a meticulous follow-up [70, 71].

27.14 Conclusion, Limitations, and Future Implications

In conclusion, the prevalence of KC is relatively high worldwide, with a special increase in prevalence and incidence among the Asian communities. However, this prevalence suffers a considerably high range of variation. It can be noted that such prevalence is increasing with moving geographically toward the east. Nevertheless, there is still a need for conducting community-based rather than hospital-based studies to assess the actual prevalence and incidence of KC. There are a number of potentially associated risk factors for KC. These factors may include genetic profile, consanguinity, geographic latitude, exposure to direct light (especially UV), and personal behaviors (such as eye rubbing). KC is one of the indirect causes of low vision and blindness (as it leads to refractive errors), and, hence, it is one of the areas for improvement in reduction of the global magnitude of low vision and blindness due to the outstanding effect of refractive errors in visual impairment. Genetically, there is a number of associated genes and SNPs that have been shown to be associated with KC. Various studies have indicated that it is quite possible that there are more than one gene contributing to the development of KC. This, of course, is in addition to other factors. The search continues for more genes and SNPs associated with KC.

Meanwhile, management of KC is now available and accessible. However, some of the cases may either present late to eye care services or even never sought such services while there are no early detection and referral mechanisms in place yet. In terms of treatment modalities, there is a wide range of available options; however, the crucial issue is that the selection of the most appropriate option should be evidence based and built on specific clinical criteria aiming to increase the probability of success. Some of these optional are temporal in terms of having no effect on the progress of the diseases, while some others are advanced enough to stop such progression. However, it would need specific surgical knowledge and skills in addition to availability of sophisticated instruments.

There is a rapid increase in the volume and quality of research in this area. Since the introduction of high-quality diagnostic and interventional instruments that can easily, quickly, and accurately assess the corneal topography and intervene with a very sophisticated and less invasive way at the exact targeted depth of the cornea, the ceiling of expectations for a successful intervention is increasing on daily basis. Improvement in surgical procedures such as cross-linking interventions is a concurrent area for clinical and experimental research. Thus, there is almost a new horizon every day in this field. It is anticipated that in the near future, there would be a highly effective resolution of KC which would both correct vision, stop, and even revers the progressions of the disease.

Declaration of Interest The authors report no conflict of interest.

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Genetics of Myopia

28

Sushil Kumari Sangwan, Arundhati Sharma, Namrata Sharma, and Radhika Tandon

Abstract

Myopia a common cause of blindness has become a challenging problem due to its frequency and complications and is expected to increase by the end of the decade. The prevalence estimates vary in different populations, age groups, ethnicity and geographic regions. In spite of studies and surveys by World Health Organization (WHO), National Health and Nutrition Examination Survey (NHANES), Refractive Error Study in Children highlighting its seriousness there is dearth of well-designed studies to tackle this problem. Though recent findings identify environmental and genetics factors having a causative role with a number of myopia genes being identified by linkage analysis and Genome Wide Association Studies, the underlying pathological mechanisms of this complex problem are yet unexplored. Understanding the genetic basis might lead to improvement in onset prediction, treatment and prevention and in turn to effective therapies to the myopia epidemic.

Keywords

Myopia • Blindness • Genetics • Genes • Association • Refractive error • Axial length

28.1 Introduction

Myopia or near or short sightedness can be classified as (1) juvenile-onset myopia which develops and progresses between ages of 10 and 16 years [1] and (2) pathologic or high-grade myopia which usually begins to develop during perinatal period and is connected with rapid refractive error myopic shifts before 10–12 years of age. It is a notable reason for legal blindness in many developed countries [1, 2].

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28.2 Epidemiology

Myopia affected 1.6 billion people worldwide in 2000 which is expected to increase to 2.5 billion by 2020. Universally, it affects about 1–2% of the population, approximately one-third of the US population and over 90% population of some East Asian countries [3].

According to a recent World Health Organization (WHO) [4] report, 14 subregions of the world had an estimated 158 million cases with distance vision impairment, with 61.9 million in the Western Pacific Region and followed by 54.5 million in the Southeast Asia Region. The estimated global gross domestic product loss owing to distance vision impairment was US $202 billion annually with a drastic increase in the last two decades in comparison to the statistics reported earlier [5].

There is variability in prevalence estimates from different countries depending upon the population, age group, ethnicity and geographic regions studied.

In 2004, among 12–54-year-olds in the United States, 42% were affected, a figure which has nearly doubled in the prior 30 years [3]. In August 2008, the National Health and Nutrition Examination Survey (NHANES) reported that 33.1% of the US population is affected with myopia, with higher prevalence in younger than older, in females than in males and in Caucasians than Mexican Americans or African Americans [6].

The prevalence of myopia is most frequent in school-going age, particularly high in college and university students, albeit it rarely occurs in less educated populations [7]. Among school-aged children of urban centres of Asia, the myopic prevalence approaches nearly 40%, with much higher rates reported among more educated and older students.

On the basis of a common Refractive Error Study in Children protocol [8], 5-year-old children from various countries and cultures have showed very few refractive errors and that the same children depending on schooling and learning systems develop myopia and stand apart from a low percentage in Nepal to about 70% in China [9].

Recent reviews suggest a positive association between myopia and high intelligence, though the link between the two is missing and is confounded by a number of factors, but evidence suggests contribution of environmental and genetic factors [10].

In spite of a number of large-scale population-based prevalence studies from Southeast and East Asia, there is dearth of data from well-designed longitudinal cohort studies [11].

28.3 Asian Perspective

Though the Caucasian Americans are the primarily affected ethnic race worldwide [6], prevalence of myopia is particularly high in those of South Asian descent [12], and in some East Asian countries, the prevalence of myopia exceeds 70% among teenagers and young adults [13]. Rapid changes were first noticed in Inuits in North America as the populations moved into settlements, [14] but it has been best documented in different population-based birth cohorts of Singapore [15] and China (Taiwan [13, 16] and Guangzhou [17]).

In terms of major population genetic clusters [18], the prevalence of myopia varies highly as per the locations of children of each European, South Asian and East Asian population clusters, with rural areas generally having lower prevalence than urban areas.

On the grounds of rural-urban environment or location, the prevalence differences of myopia have been confirmed further, with inner-city urban areas on higher side in comparison to outer suburban areas. This data implicated clearly that small to moderate changes in environment may affect development of myopia, even within a common predominantly urban area [19].

The preponderance of myopia is generally low in children growing up in Africa but is higher for those growing up in the United States or United Kingdom of Sub-Saharan African ancestry [20].

In Singapore [15, 21], the prevalence has increased at speed in all three major ethnic groups (Chinese, Indians and Malays) since 1987–1992 [6, 22], suggesting that rapid change in these ethnic groups has been mainly caused in all children of Singapore by myopigenic social environmental factors to which they are exposed to.

In addition, outdoor activity is recognised as a key environmental determinant of myopia. In children of both Singapore and Australia, the total time spent outdoors was associated with less myopic refraction, independent of other factors like indoor activity, reading, and engagement in sports [23]. A comparative study of Chinese children in Singapore and Sydney also showed a protective effect of outdoor activity [24].

Overall, as per recent findings, the prevalence of myopia seems to depend on where children grow up and the environments to which they are exposed, rather than aspects of genetic ancestry.

28.4 Aetiology

The aetiology of myopia is a matter of historical dilemma for centuries. Five decades ago, myopia was believed to be genetic, with minor environmental influences, but since then, there has been a debate between nature (biological) and nurture (environmental) on the causes of myopia. Both inherited as well as congenital forms of myopia have been documented. But in late 1970, experimental studies showed that changes in visual experiences can induce myopia by promoting eye growth [25]. Although the underlying mechanism is still emerging, but its effect is to coincide the plane of the retina with the focal point of the eye's optical system by means of adjustments in eye growth. In this paradigm, altered visual stimuli (i.e. optically blurred images) start a cascade of signals that originates locally in the sensory retina, traverses the retinal pigment epithelium and the vascular choroid and finally regulates the growth of eye via active remodelling.

This mechanism may be disrupted by the changes in sequence of biochemical events and lead to refractive errors which in turn results in blurred vision. Hence, any gene that has a role in this complex signalling pathway may contain susceptibility variants for myopia [26].

The aetiology of myopia is heterogeneous. The Online Mendelian Inheritance in Man (OMIM) database listed 261 genetic disorders in which myopia is one of the symptoms, and this list includes the syndromic form of high myopias. In connective tissue disorders like Marfan and Stickler syndromes, and complete and incomplete congenital stationary night blindness, high myopia is associated with other symptoms which define the disease, whereas in cases of non-syndromic high myopia, the main clinical feature is high, familial, early-onset myopia, but the appearance of myopia during the middle childhood years is commonly known as school myopia [27].

Presently, there are number of evidences which highlight the genetic contributions for the existence of high myopia, although these might be reduced in younger cohorts given the increasing preponderance of acquired high myopia in East Asia. Contrary to this, it seems that school myopia is multifactorial, possibly involving a small effect from number of genes and a major contribution of environmental factors.

28.5 Pathology

Myopia still remains an unsolved medical problem though correction to optical errors can be done by optics or surgical procedures. It frequently predisposes the affected to other ocular morbidities like glaucoma, macular choroidal degeneration, cataracts, and retinal detachment which are main causes of visual loss and blindness [2, 6]. Nevertheless, the scarcity of data in relation to underlying pathophysiological molecular mechanisms has not permitted adequate diagnosis, prevention or treatment.

Currently, evidence from research on genetic loci indicate that this complex problem of

myopia and its progression totally depend on genetic and environmental factors. Children of affected parents may be more likely to get the condition. In addition, the condition is environmentally driven in genetically susceptible individuals. Over the last three decades, a remarkable rise in the prevalence of myopia worldwide and associations of refractive error with a host of environmental factors and behavioural characteristics [28] points to a strong environmental influence on refractive errors. Nevertheless, a strong association of myopia with parental history as well as heritability studies [29] has consistently indicated that >50% of the variability of refractive error within populations is determined by genetic factors.

28.6 Genetic Aspects

Myopia causes substantial ocular morbidity, and its annual economic burden as per 2009 survey accounts for $139 billion in the United States and $268 billion worldwide [30]. High-grade myopia, a leading cause of blindness, is associated with retinal detachment, macular degeneration and glaucoma [6, 31]. Various studies have documented an autosomal dominant inheritance and show 30% of the cases to have a positive family history [32].

Heritability estimates are high for ocular refraction ranging from 50% to more than 90% [33].

Familial aggregation studies have estimated sibling recurrence risks (λs) for refractive errors to range from 2 to 5.61 in myopia [34]. Extreme refractive errors show greater familial aggregation than milder forms [35]. Children of myopic parents tend to have longer eyes [36] and are more likely to develop myopia during childhood or adolescence [37].

Segregation analyses of population-based samples for ocular refraction are consistent with complex inheritance involving several genes and/or shared environmental factors [38].

Familial refractive errors occur in simple (non-syndromic) forms or are accompanied by other systemic or ocular abnormalities. Syndromic refractive errors are either monogenic or oligogenic and occur with a wide spectrum of clinical presentation. Myopia is reported in numerous ocular syndromes which include X-linked and autosomal recessive congenital stationary night blindness (CSNB), X-linked retinitis pigmentosa 2 (RP2) and Bornholm eye disease (BED). The myopia in X-linked ocular syndrome appears to be secondary to mutations in loci involved in retinal photoreceptor function (NYX, RP2, MYP1). Myopia is a characteristic feature in heritable connective tissue disorders like Knobloch syndrome (COL18A1 mutations), Marfan syndrome (FBN1 mutations), and type 1 and type 2 Stickler syndrome (COL2A1 and COL11A1 mutations, respectively) [39].

Interestingly, two independent studies [40] show statistical associations of simple myopia with COL2A1, suggesting its role in non-syndromic refractive errors. Young et al., in 1998, mapped the first genetic locus MYP2 for non-syndromic high myopia to chromosome 18p11.31 [41]. Subsequently, the list of myopia loci increased to 16 which include MYP2–MYP17. Seven loci for refractive phenotypes (MYP1, MYP3, MYP6, MYP11, MYP12, MYP14 and MYP17) were successfully replicated in independent linkage studies [42–46], and mutations in CTNND2 (cadherin-associated protein) of MYP16 loci were identified for high myopia [47]. Most of the loci for refraction traits were identified in families who segregated high myopia consistent with autosomal dominant modes of transmission [41, 45].

Functional and positional candidate gene approaches have shown associations with genes involved in extracellular matrix (ECM) growth and remodelling pathways. These include collagens [(COL2A1 [40], COL1A1 [[40])], transforming growth factors (TGFB1 [48], TGFB2 [49], TGIF1 [50]), the hepatocyte growth factor (HGF [51]) and its receptor (CMET [52]), insulin-like growth factor (IGF1 [53]), matrix metalloproteinases [54] (MMP, MMP2, MMP3 and MMP9) and the proteoglycan lumican (LUM [55])]. Later a meta-analysis suggested lack of

association between lumican (LUM) gene promoter region rs3759223 polymorphism and high myopia risk [56].

Of particular interest are the collagens, which constitute over 90% of the mammalian sclera [57] and undergo active growth and remodelling as shown in animal models of myopia. The results provide strong evidence that the genetic basis for human refractive error may partially be explained by variations in genes that directly affect ECM composition in the scleral tissue, leading to differential rates of ocular enlargement and differences in susceptibility to myopia [58].

Evidence obtained from epigenetic studies on mice suggests hypermethylation of CpG sites in COL1A1 promoter may inhibit scleral collagen synthesis and lead to development of myopia [59].

HGF [51] and COL2A [40] have also shown positive association with the milder refraction phenotypes [51]. These and other studies on genetic associations between ocular refraction and polymorphisms in matrix metalloproteinase genes (MMP1, MMP2, MMP3 and MMP9) [54] suggest a common biological pathway for extreme and mild myopia, as MMPs which regulate cell-matrix composition by cleaving a number of ECM constituents interact biologically with gene products known to be related to refractive phenotypes [60].

Multimarker fine-scale linkage disequilibrium methods indicated statistical association of refractive error with three genes on long arm of chromosome 3: MFN1, PSARL (or PARL) and SOX2OT [61]. Nakashiki et al. [62] identified a polymorphism (rs577948) at 11q24.1 adjacent to the BLID gene that was associated with an elevated risk of pathological myopia (odds ratio = 1.37) in a Japanese group. MFN1, PSARL and BLID are expressed in mitochondria, where MFN1 is involved in mitochondrial fusion and PSARL and BLID are involved in mitochondria-led apoptosis. The mechanism by which mitochondrial programmed cell death can lead to myopia is not yet elucidated, but this is a promising area for future experimental studies in myopia [63].

Genetic variations of ocular axial length-related genes (ZC3H11B, RSPO1, GJD2) are associated with high myopia susceptibility in Han Chinese population, but their functional role in pathology is still unclear [64].

In spite of identification of numerous myopia loci, there are no known genes for non-syndromic myopia. GWAS studies have involved either high-grade 'pathological' myopia case–control studies or analysis of quantitative 'healthy variation' of refractive error using population-based cohorts. The first high myopia GWAS study in 2009 identified a susceptibility locus on chromosome 11q24 for BLID (inducer of apoptotic cell death) and LOC399959 (function not known yet) genes [62]. Later in 2010, two different GWAS studies identified a single locus each on chromosome 15q14 near the GJD2 gene and 15q25 near the RASGRF1 gene [62, 65]. Both genes are highly expressed in the retina and are implicated in myopia development in animal models. GJD2 encodes a neuron-specific protein (connexin36) found in retinal photoreceptors, essential in the transmission of rod-mediated visual signals, and RASGRF1 encodes a Ras protein-specific guanine nucleotide-releasing factor1, and its expression activate Ras protein which has functional influence on myopia pathogenesis [66] is regulated by muscarinic receptors [67] and retinoic acid. Moreover, muscarinic acetylcholine receptor 1 gene (CHRM1) polymorphisms were already reported to be associated with high myopia in Taiwanese population [68].

Polymorphisms of RASGRF1 gene (rs8027411) showed lower risk [69] and PAX6 gene (rs662702, rs3026393, rs644242, rs3026390 and rs667773) showed higher risk of high myopia in Japanese population [70].

Concurrently, the Consortium for Refractive Error and Myopia (CREAM) and DNA testing company 23andMe confirmed two reported loci and discovered 20 new genes in association with myopia [71, 72]. Remarkably, 16 of the 20 novel loci identified by Kiefer et al. 2013 were confirmed by CREAM, and out of the 22 loci discovered by the CREAM analyses, 14 were replicated by 23andMe, and the regions not

confirmed had suggestive associations in cascade of retina-to-sclera signalling [26]. Despite these discoveries, in common with most complex diseases, the significant associations only explained 3–4% of the variations. Further, GWAS meta-analyses have identified RBFOX1 on chromosome 16 as a candidate gene for refractive error susceptibility in European populations, [73] and GWAS have identified genetic variants in high myopia studies in Chinese populations [74]. Exome sequencing holds promise of identifying genes, particularly in high myopia subjects (like ZNF644 mutations) [75] and in families with dominantly inherited high-grade myopia (like SCO2 and LRPAP1 mutations) [76, 77], though the relevance of these findings remains uncertain. But a recent study demonstrated no association between heterozygosity for pathogenic SCO2 variants and high-grade myopia in either human patients or in mice [78].

Therefore, the available evidence suggests heterogeneity for high myopia while leaving open the question of whether there is any overlap between genes that might be responsible for both the rarer forms of high myopia and more common, less severe juvenile onset myopia.

But there is limited success even after years of attempt in identification of myopia genes by linkage analysis and by GWAS studies.

The identification of myopia susceptibility genes will not only provide insight into the molecular basis of this significant eye disorder but will also identify pathways involved in eye growth and development. In addition to enhancing our understanding of the underlying biology of myopia, a better understanding of genetic factors in myopia might lead to improvement in predicting the onset, treatment and, perhaps, prevention. This effort may lead to effective therapies to treat or potentially prevent this common eye condition.

A future area of interest in myopia research is to understand the interaction between associated genes and environmental effects. Through these and other ongoing efforts on the novel gene-based tests and therapies for common ocular disease may help reduce the global burden of visual impairment.

28.7 Summary

Myopia is a common but complex multifactorial and genetically heterogeneous ocular condition with a significant visual morbidity and impact on global public health. It has become a challenging problem, due to its high incidence and complications of blindness related to high myopia as focused by epidemiological data. Several recent genetic studies investigated and identified numerous genetic variants on different chromosomes associated with axial length and myopia/refractive error or in pathophysiological pathways using different approaches. Both genetic and environmental factors (like near work, education, diet, physical and outdoor activities, etc.) have been associated with the causation, development and progression of myopia. Moreover, recent studies have identified a significantly larger genetic basis for myopia in subjects with a higher level of education.

Recent changes in myopia incidence do not obviously result from short-term shifts in the genetic makeup of the population. Instead, secular trends in environmental and behavioural factors are thought to be driving the myopia 'epidemic' throughout the world.

An improved understanding of the predisposing genetic factors allows for enhanced screening modalities and paves the way for translation of genome technology into the clinics. In spite of these, complete prevention of myopia remains a long-term goal, and current clinical as well as genetic research is aimed at slowing the progression of the disease or delaying the onset.

This review was aimed to provide an overview of the genetics on myopia available in literature, till date, and also to unravel the contribution of genetics in complex ocular disorders. Though it has raised questions that remain unanswered, eye care practitioners and parents can potentially learn about the methods

that may ultimately improve a child's quality of life or lower the risk of sight-threatening complications.

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Ocular Implications of Gaucher Disease 29

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Abstract

Gaucher disease (GD) has been reported in almost all the regions of Asia. Although ophthalmic manifestations of GD include corneal changes, retinal pigment epithelium (RPE) atrophy, and lesions at the vitreoretinal interface, most published reviews of GD do not cover the eye, and ophthalmic ultrastructural reports on GD are scarce, lack complete survey of all ocular tissues, and do not capture its heterogeneity. Variability of clinical manifestations does not forecast the disease's wider subcellular heterogeneity. We describe the ophthalmic histopathological, immunohistochemical, and ultrastructural changes in two autopsied patients with type 1 GD. Gaucher body inclusions (GBIs) were present in the ciliary body, retinal ganglion cells, choroid, and sclera, although absent elsewhere in the eye. GBI accumulation varied among ocular tissues in morphology, number, and volume. The morphology of GBIs in the choroid and sclera differed from those in the ganglion and ciliary body cells. While GBIs accumulated in large numbers, occupying much of the cytoplasm in cells of the choroid and sclera, there were fewer in the ganglionic cell layer and ciliary body. The choroid and sclera served as a sink, accumulating GBIs or their contents originating from other ocular tissues. The mechanism underlying this high GBI concentration in these specific regions remains unknown. We postulate that in addition to the hematogenous route, mobile retinal microglial cells may play a role in the localization of GBIs in the eye. Such an intraocular movement pathway might have pharmacokinetic ramifications for intravitreal drugs, intraocular gene therapy vectors, and possibly vitreoretinal tamponade agents (e.g., silicone oil). Furthermore, the quantity, density, size, and shape of the

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GBIs differed in the two patients studied, with a higher concentration of GBIs seen in the patient with the more severe Gaucher genotype (N370S/c.84insG), reflecting the heterogeneity of GD1 at the ultrastructural level.

Keywords
Gaucher disease • Gaucher cell • Gaucher body inclusion • Electron microscopy • Immunohistochemistry

29.1 Introduction

Gaucher disease (GD), the most common inherited lysosomal storage disorder [5], is caused by autosomal recessive mutations in *GBA1*, the gene encoding the lysosomal enzyme glucocerebrosidase [2, 5, 38, 39]. The majority of the more than 300 different mutant *GBA1* alleles identified in GD are missense mutations causing a decrease in the catalytic function and/or stability of the enzyme [18]; however, what is still unresolved is the exact mechanisms by which the enzymatic defect causes GD [20]. GD has been reported in almost all the regions of Asia [6, 13, 21, 23, 31, 42, 48, 49, 51, 53]. While it is a global panethnic disorder, GD is more frequent among Ashkenazi Jews where the carrier frequency is approximately 1 in 15.

GD is a clinically heterogeneous disorder that is conventionally classified into type 1 (non-neuronopathic disease), type 2 (acute neuronopathic disease), and type 3 (chronic neuronopathic disease) [5]. However, there is considerable heterogeneity among patients with the same Gaucher type, including ultrastructural differences within the various tissues of the eye, liver, and bone marrow. Ultrastructural examination with electron microscopy of the bone marrow, liver, skin, or spleen is rarely used for diagnosis of the disease.

Type 1 GD accounts for a majority of GD, does not involve the central nervous system, and has a wide spectrum of presentations [7, 45]. GD1 includes asymptomatic patients, those with splenomegaly and thrombocytopenia, and others with orthopedic complications or more profound blood dyscrasias [7, 45]. Type 2 (GD2) is the most severe form of GD. It has an onset within the first year of life and is progressive and characterized by hepatosplenomegaly and progressive central nervous system involvement. Life expectancy is less than 2 years [52]. Type 3 (GD3) has attenuated neurological features compared to GD2 and typically manifests with a specific oculomotor finding, slowing of the horizontal saccades; it is often accompanied with visceral involvement [45, 46].

Neurological features including Parkinson disease and peripheral neuropathies have been identified in patients with GD1 [1, 3, 27, 29]. This has led to the suggestion that GD may be a continuum of phenotypes rather than distinct subtypes [38, 45].

Glucocerebrosidase is required for the degradation of glycosphingolipids. Deficiency of glucocerebrosidase results in the accumulation of glucocerebroside (glucosylceramide) within cells of the mononuclear phagocyte system, particularly those in the liver, spleen, and bone marrow [2, 5]. In addition to the enzyme deficiency, it is thought that altered inflammatory cytokine profiles, bioactive sphingolipid derivatives, and alterations in the microenvironment play a role in disease pathogenesis [45].

Since the 1990s, enzyme replacement therapy (ERT), with drugs like imiglucerase (Cerezyme; Genzyme Corporation, Cambridge, MA), has resulted in dramatic improvement in the hematological and visceral disease [45, 54]. Conventionally, it is assumed that ERT does not pass the blood-brain barrier [43, 54]. Progression of preretinal and retinal pigment epithelium (RPE) lesions in patients with GD1 and GD3 receiving imiglucerase therapy has been reported [12, 50].

Thus, Coussa et al. have proposed that ERT molecules cannot cross the blood-retinal barrier [12].

Ocular involvement in GD is well recognized. Anterior segment manifestations in GD1 include pinguecula-like lesions, corneal haze, increased corneal thickness, and abnormal keratocytes in the corneal stroma [16, 35]. The observed pinguecula-like lesions were found to contain Gaucher cells [35]. Posterior segment manifestations include vascular leakage, occult choroidal neovascular membranes, retinal artery occlusions, epiretinal membranes, and macular retinal pigment epithelium (RPE) atrophy [4, 17, 36, 50]. Oculomotor apraxia was reported in GD3 patients [44]. Optical coherence tomography (OCT) of the retina has demonstrated preretinal perivascular white globular lesions both in patients with GD1 and GD3 [12, 19]. Thinning of the ganglion cell complex (i.e., distance between the retina's internal limiting membrane and the inner nuclear layer) in GD1 patients has also been reported [30]. Electroretinograms (ERGs) have demonstrated a reduced scotopic B-wave response in GD1; this may be due to accumulation of glucosylceramide in the retinal glial cells [37].

It is thought that the ocular abnormalities in GD result from the Gaucher cells, namely, macrophages engorged with sphingolipid resulting in a "crumpled silk" appearance. It has since been proposed that the glucocerebroside also accumulate within retinal ganglion and glial cells [8, 9, 12, 35, 37]. However, the newly recognized associations with multiple myeloma and Parkinson disease in GD are prompting a rethinking of the traditional macrophage-centric view of the pathophysiology of GD [45].

In this chapter, we describe the ophthalmic manifestations of GD1 in two deceased individuals with GD1; this encompasses histology, immunohistochemistry, and electron microscopy and speculates on the pathophysiology of the disease within ocular tissues particularly the localization of glucocerebroside and its movement between ocular tissues, as well as its accumulation as Gaucher body inclusions (GBI).

29.2 Clinical Summaries

Patient One

This 61-year-old Ashkenazi Jewish female with GD1 presented at age 14 with painful bone crises. Assays performed on her fibroblasts revealed deficient glucocerebrosidase. Genetic testing, conducted by sequencing all exons of *GBA1* [40], revealed genotype N370S/c.84insG. She underwent splenectomy and bilateral hip replacements. Ultimately, she was treated with high-dose intravenous imiglucerase but had an interruption in her therapy in 2010, due to a global shortage of ERT. From the age of 57, she developed progressive cognitive decline with memory loss, hallucinations and paranoia, diagnosed as dementia with Lewy bodies (DLB). An MRI of the brain revealed diffuse cerebral atrophy. Her neurological function deteriorated rapidly and she died of pneumonia in 2014. At autopsy, extensive Lewy body pathology was found in her central nervous system (cortex, hippocampus, substantia nigra, and celiac ganglia), and Gaucher cells were seen in the liver, lungs, and bone marrow. Her father also died with the diagnosis of DLB.

Patient Two

This 73-year-old Ashkenazi Jewish female with GD1 was diagnosed at the age of 47 when a biopsy revealed that 60% of her bone marrow has been replaced by Gaucher cells. Her fibroblasts showed deficient glucocerebrosidase activity, and genetic testing revealed homozygosity for mutation N370S. In addition to GD1, she also suffered from Sjögren's syndrome, parkinsonism, mixed connective tissue disease, vasculitic skin lesions, cryoglobulinemia, polyclonal gammopathy, aseptic necrosis of the femur, and chronic fatigue syndrome. Her GD had been treated for 4 years with imiglucerase, and she also received plasmapheresis and erythropoietin. In her last year of life, she had a chronic hospitalization for ascites, renal failure, chronic granulomatous pulmonary disease, and Lewy body dementia. She died in 2014 from pneumonia and multiple organ failure and was found to have metastatic ovarian cancer. At

autopsy, Lewy body pathology was found in her central nervous system, but no Gaucher cells were found in her liver, spleen, or bone marrow.

29.3 Materials and Methods

We examined all four autopsied eyes from the two patients with GD1. Routine histopathology and immunohistochemistry were performed. Immunohistochemistry was done using primary antibodies against CD68 (to localize macrophage and many microglia cells), CD20 (to localize B cells), and CD3 and CD45RO (to localize leukocytes, mainly activated and memory T lymphocytes). All monoclonal antibodies were purchased from Dako (Dako North America, Inc., Carpinteria, CA, USA). The secondary antibody used was biotinylated goat anti-mouse IgG (1: 200; Vector Laboratories, CA, USA).

Nine specimens from the two patients underwent further examination by electron microscopy. Ultrastructural specimens from Patient One included the OD cornea, ciliary body, retina, choroid, sclera, optic nerve and OS cornea, corneal limbus, ciliary body, retina, choroid, and sclera. Patient Two's OD choroid and sclera were examined ultrastructurally. Transmission electron microscopy (TEM) was carried out according to Ogilvy et al. [32].

29.4 Results

29.4.1 Histology and Immunohistochemistry

Patient One

Both eyes showed similar pathology. A few foamy and plump basal epithelial cells were present within the corneal epithelial cell layer, and focal calcium deposition was seen at the peripheral Bowman's layer (Fig. 29.1a). The ciliary body epithelia contained few granular, vacuolated macrophages, most of which were CD68 positive (Fig. 29.1b, c). The vessels of the retina were moderately sclerotic. Occasional granular macrophages (CD68 positive) were found in the inner retina of both eyes. Numerous large granular, vacuolated macrophages some with crumpled silk appearance were present in the choroid of both eyes (Fig. 29.1e, f); most of these cells were strongly CD68 positive (Fig. 29.1g) and were considered Gaucher cells. A few T cells (CD3 positive and CD45RO positive), and rare CD20 B cells were observed. There were mildly sclerotic choroidal blood vessels. The sclera contained large granular vacuolated CD68-positive macrophages mainly surrounding the posterior ciliary vessels (Fig. 29.1d–i) that were also Gaucher cells, a few T cells, and occasional B cells (CD20 positive). The optic nerves revealed mild atrophy (Fig. 29.1h) and infiltration of CD68-positive macrophages (Fig. 29.1j).

Patient Two

Both eyes shared similar pathology. The corneas showed focal, abnormal, and duplicated corneal epithelial basement membranes. The choroid contained sclerotic vessels in some areas and a few Gaucher cells, appearing as large cells with heterogeneous granular vacuolated cytoplasm and nuclei that were partly displaced to the side (Fig. 29.1k). These cells were mostly CD68 positive (Fig. 29.1l, m). A few isolated macrophages were also seen in the retina. The right optic nerve showed mild gliosis.

29.4.2 Transmission Electron Microscopy

Patient One

No Gaucher body inclusions (GBIs) were observed in the cornea of either eyes. In the basal cells of the corneal epithelium, loose infoldings, digitation, degenerate mitochondria, numerous vacuoles, and apoptotic nuclei were observed (Fig. 29.2a). Some cells contained fine granular-fibrillar material without cytoplasmic organelles. Bowman's layer was disorganized and disconnected from the underlying corneal stroma. Stromal collagen fibers were poorly

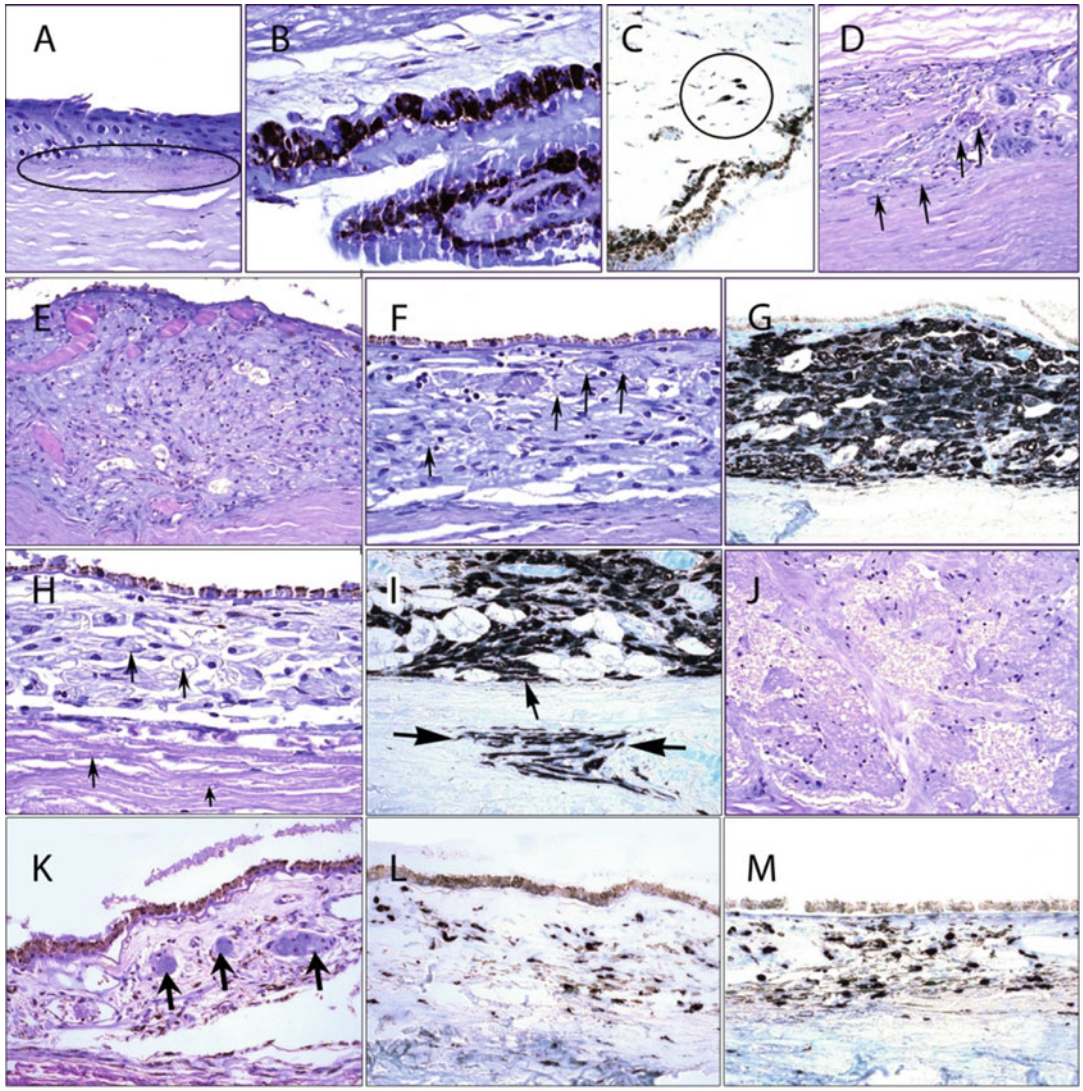

Fig. 29.1 (**a–j**), from Patient One; (**k–m**), from Patient Two. (**a**), cross section (CS) of OS cornea showing foamy, plump, swollen basal epithelial cells, and focal calcium deposits (*circled*) at the peripheral Bowman's membrane both nasally and temporally. (**b, c**), CS of OD ciliary body showing histiocytes; (**b**), the outer pigmented epithelium and, (**c**), CD68-positive histiocytes in the nonpigmented ciliary epithelium (some marked by a *circle*). (**d**), CS of sclera showing some foamy histiocytes (*arrows*). (**e–g**), CS of choroid, (**e, f**), (**h**, **e**) staining showing foamy macrophages throughout the OD choroid (*arrows* in **f**); (**g**), immunohistochemistry staining of the choroid showing CD68-positive cells. (**h, i**). CS of the choroid and sclera. (**h**). The choroid and sclera showing foamy macrophages (arrows); (**i**). CD68-positive histiocytes in the choroid and sclera (*arrows*). (**j**), CS of the OD optic nerve showing atrophy in the neuronal bundles. (**k–m**), from Patient Two. (**k**). CS of OD choroid showing GBIs (*arrows*). (**l, m**), immunohistochemistry staining with CD68-positive cells in the choroid of OD and OS, respectively

aligned. The corneal stroma also contained degenerate keratocytes and melanosome-laden macrophages (Fig. 29.2b), but no GBIs were seen.

The right ciliary body contained GBIs (arrow in Fig. 29.2d). Within the ciliary pigmented epithelial cells, melanosomes were normal but had unusually large vesicles (arrow in Fig. 29.2c,

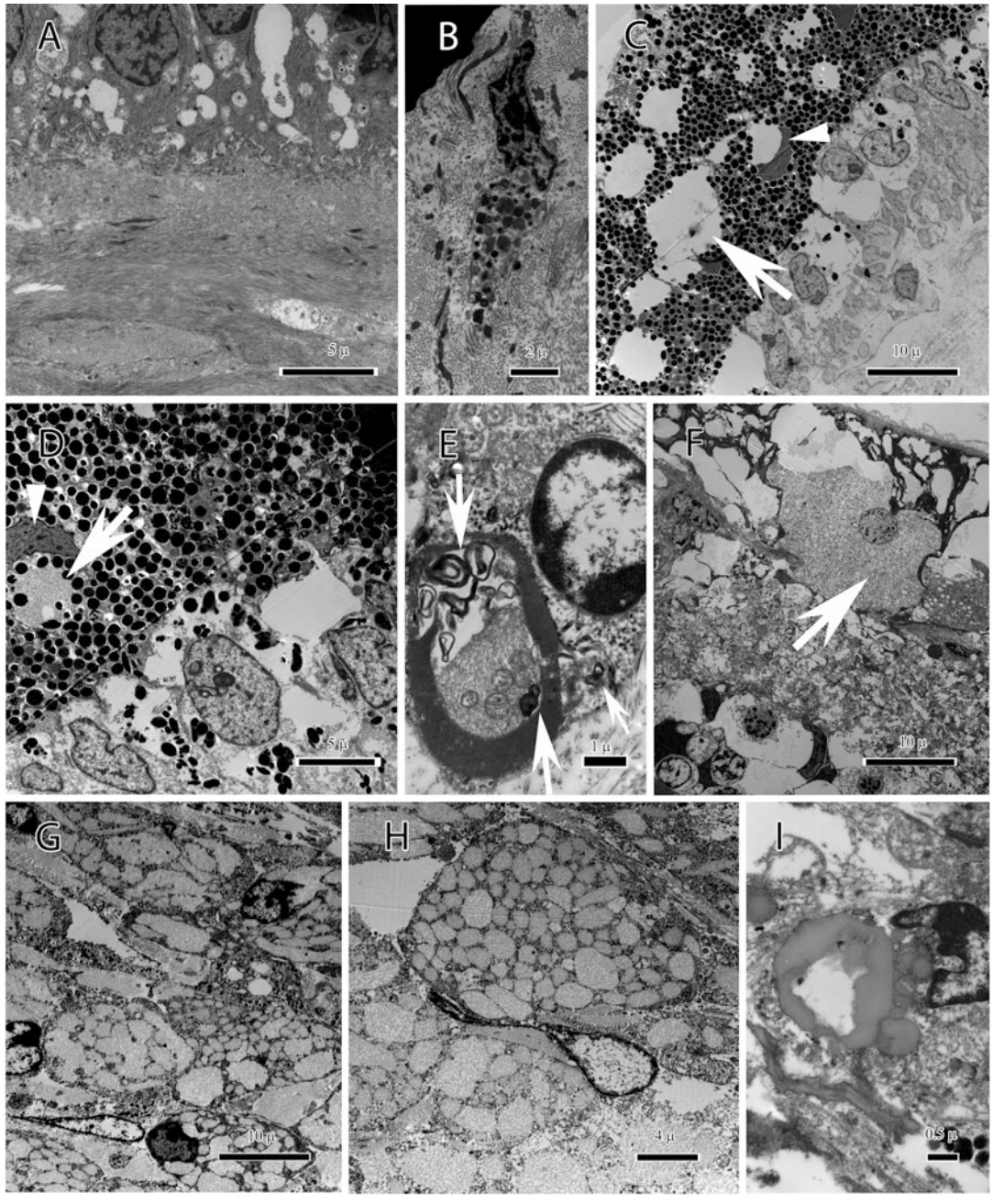

Fig. 29.2 Transmission electron micrographs of ocular tissues from the two patients with Gaucher disease. (**a–h**) from Patient One; (**i**) from Patient Two. (**a**). Cornea, (**b**). Melanosome-laden macrophage in the cornea, (**c**). CS of ciliary body showing the empty Gaucher body inclusions in the pigmented epithelium (*arrow*) and apoptotic nucleus (*arrow head*), (**d**). CS of ciliary body showing Gaucher body inclusion (*arrow*) and compressed nucleus (*arrow head*), (**e**). CS of a nerve axon within the ciliary body showing degenerate myelin sheath forming inclusions within the axon's lumen and inside Schwan cell (*arrows*), (**f**). Ganglionic cell with Gaucher body inclusion, (**g**). Choroid with many Gaucher cells, (**h**). Higher magnification of Gaucher cells in the choroid, (**i**). Gaucher body inclusions within the choroid of Patient Two

probably remains of GBIs) and shrunken apoptotic nuclei (arrowheads in Fig. 29.2c, d). The ciliary nonpigmentary epithelium appeared vacuolated and degenerate. Nerve axons had abnormal degenerate myelin sheaths, numerous membranous myelin inclusions and debris, shrunken and dense axoplasm, and degenerate mitochondria (Fig. 29.2e). The surrounding Schwann cells contained myelin debris, degenerate mitochondria, and cholesterol crystals. Degenerate fibroblasts, melanocytes, and melanosome-laden macrophages were also present. The left ciliary body had no GBIs in the ciliary pigmented and nonpigmented epithelia, and the latter appeared vacuolated and degenerate.

The neural retina was degenerate. The nerve fiber layer consisted mostly of degenerate glial cells. Also within the inner retina, some neuronal cells within the ganglion cell and inner nuclear layers were found to contain fine homogenous granular material, assumed to be glucocerebroside, which replaced the normal cytoplasmic intracellular organelles (Fig. 29.2f). Blood vessels within the inner retina appeared abnormal with many splits in their basement membranes.

The RPE cells were degenerate and without GBIs, although remnants of vacuoles were present in the left RPE. Bruch's membrane appeared condensed with loss of elastin fibrils in the right eye and contained many electron dense deposits in the left eye.

Choroidal stromal collagen fibers were found in various stages of degeneration (Fig. 29.2g, h). Abnormal cells were also found within both choroid and sclera, which were completely filled with GBIs. These GBIs had almost totally replaced the intracellular contents, causing nuclei to be eccentrically positioned and sometimes compressed against the cell membrane. Although little cytoplasm remained, some spaces did persist between the GBIs. In these areas, degenerated mitochondria and occasionally melanosomes were found (Fig. 29.2g, h). These choroidal and scleral GBIs were filled with granular material that varied in size, shape, and number. The smaller GBIs appeared to have fused with one other to produce the larger GBIs.

Overall, all GBIs in the ocular tissues of this patient had the same density and grainy appearance.

Patient Two

The ocular tissues were slightly degenerated due to autolysis; however, ample features were preserved. In the right eye, RPE was degenerate and most of its melanosomes had less electron density and with mitochondrial breakdown evident. No GBIs were seen in the RPE. Bruch's membrane was thickened and composed of dense elastin deposits in the lower two thirds due to elastin fiber disintegration. In the choroid, a few Gaucher cells were found containing numerous GBIs filled with homogenous light electron dense material; some of the GBIs were very large (Fig. 29.2i). The choroidal fibrocytes were degenerate; and surrounding choroidal collagen fibrils were abnormal in appearance with low density and rough margins. A few bacteria were also found embedded between the choroidal collagen fibrils. The sclera contained abnormal collagen, degenerate nerves, and degenerate fibrocytes.

The GBIs in the two patients were found to differ in quantity, density, size, and shape. This was most evident in the GBIs within the choroid and sclera. Patient One's GBIs were smaller and more granular. Patient Two's GBIs were larger and consisted of smoother homogeneous material. There was a lower GBI density in the choroidal and scleral Gaucher cells in Patient Two. These differences suggest that GBIs can be composed of varying materials, thus demonstrating the heterogeneity of GD1 on an ultrastructural level.

29.5 Discussion

There are many published reports on the clinical manifestations, biochemistry, and genotype-phenotype correlation in GD, but very few focus on the ultrastructural features of macrophages and their biochemical transformation into Gaucher cells. Additionally, compared to the spleen and liver, the eyes in GD have not

been adequately studied ultrastructurally ([14, 34, 47]). This ultrastructural study enabled us to develop a hypothesis to explain the unique distribution of GBIs within the tissues of the eye.

The ocular pattern of GD seems unique compared to other organs. There is a clear differential distribution of GBIs among ocular tissues with the choroid (diffusely in the posterior choroid) and sclera (mainly surrounding the posterior ciliary vessels) having the highest concentration of GBIs within their cells (Fig. 29.2g, h). The nature of GBI accumulation in ocular tissues varies in morphology, number, and volume. For example, there are differences in GBI accumulation and appearance between the choroid and sclera in comparison to cells within the ciliary body and in the retinal ganglion cell layer. GBIs within the choroid and sclera were well defined vesicular inclusions that accumulated in large numbers, engorging the cells and reducing their cytoplasm to the absolute minimum. GBIs in the ciliary body usually appeared as a few larger vesicles that did not encompass the entire cell, while in retinal ganglion cells the GBIs usually occupied the majority of the cytoplasm, filling the cells with a granular material assumed to be the glucocerebroside, albeit without a clear enveloping membrane (Fig. 29.2f). Additionally, while almost every cell in the choroid and sclera contained GBIs, in the ciliary body and retinal ganglionic cell layer, they were present in fewer cells.

In contrast to the Gaucher cells of the spleen, which maintain an abundant cytoplasm [34], those found in the choroid and sclera showed scant cytoplasmic content between their GBIs. Also, according to Pennelli et al., the former have been reported to possess pseudopodia (villi) which were lacking in the latter suggesting the absence of phagocytic activity.

It is puzzling why the Gaucher cells of the choroid and sclera continue to act as a sink for GBIs even after they have arrived from inflammatory cells (macrophage, best recognized) via the hematogenous route and become dysfunctional with the crowding of their cells by GBIs. Is such accumulation of GBIs a passive process that continues without any input from the host cell? This ophthalmic manifestation of GD1 has not been described previously.

Surprisingly, some of the mitochondria of Gaucher cells persisted during the buildup of GBIs, and some appeared dysfunctional. Some reports have speculated active mitochondrial involvement in the formation of GBIs [14, 34]. Although the images from Fisher and Reidbord, as well as Pennelli et al. support mitochondrial involvement in the formation of GBIs in the spleen, our TEM micrographs do not show any transition of the mitochondria to GBIs. Another mitochondrial related issue is the abnormality of autophagy in GD [41]. This is interesting since the mitochondria donate their membranes to form autophagosomes [10, 11]. Storage of glucocerebroside and glucosylsphingosine is suspected to cause defective mitochondria and thus curtail autophagy, which could in turn lead to degenerative axons and be central to the pathogenesis of neuronopathic GD [41].

The classical theory of GD pathology is macrophage centric where the macrophages are the scavengers of cells with GBIs and then become persistent Gaucher cells [12, 37]. However, it seems that in the ocular tissues and particularly in the choroid and sclera, the cells laden with GBIs include these two tissues as well as macrophages, which support our premise that cells other than macrophages are involved in the storage of GBIs.

We speculate that in choroidal and scleral cells, the GBIs or their contents may arrive from other anatomical eye sites, rather than solely being produced locally by choroidal and scleral cells. The GBI content may enter the cells in small vesicles, which later fuse together to form the larger GBIs (Fig. 29.2g, h). One possible mechanism is that the GBI material may arrive via the uveal bloodstream or aqueous humor outflow through the uveoscleral pathway and then become concentrated within the choroid and sclera [15]. In a mouse model, fluorescent dextran molecules were observed to move from the anterior chamber, past the ciliary processes, and ultimately into the choroid and sclera [26]. Large accumulations of circulating

glucocerebroside in unusual organs have been suggested as an explanation [37]. Microglia are blood-borne cells that have phagocytic functions [22]. Seidova et al. suggested that the microglia phagocytose degenerate retinal neurons, leading to glucocerebroside accumulation within glial cells (Mueller cells, astroglia, and mostly microglia) and causing a reduction in the scotopic B-wave on ERG [37]. Retinal microglia are mobile and move between the inner and outer retina and RPE [22, 24, 25]. The choroid also contains various dendritic cells that function like the retinal microglia [28]. Thus, it is possible that mobile retinal microglia and choroidal dendritic cells may be partly responsible for the observed migration of GBIs within the eye. The loading also may take place through several routes simultaneously [33].

The GBIs observed differed in our two patients. Patient One, with genotype N370S / c.84insG, was diagnosed with GD1 at the age of 14, had rapid neurodegeneration and died at age 61. Patient Two, with genotype N370S/ N370S, was diagnosed at age 47 and died at the later age of 73. Ultrastructurally, the choroidal and scleral cells in Patient One had a higher GBI density which could be related to the more severe genotype.

Although grainy deposit of what appeared to be glucocerebroside was seen in the ciliary body and ganglion cells, no classical GBIs were seen in these tissues. In contrast, the choroidal and scleral cells were extremely overloaded with GBIs and were transformed into dysfunctional sinks of glucocerebroside where the accumulated lipid transformed them into Gaucher cells.

We hypothesize that the differing concentrations of GBIs within different anatomical sites in the eye of GD1 patients are due to the movement of glucocerebroside to the choroid and sclera. The white globular lesions on the retinal surface described by Coussa et al. are most likely evidence of such deposition [12]. Seidova et al. and Lee et al. maintain that activated microglia have macrophage-like functions and are mobile within the retina [25, 37]. If glucocerebroside does indeed move within the eye as described, further work is needed to elucidate the mechanisms of glucocerebroside movement between ocular tissues. Understanding and manipulating intraocular transport pathways might have pharmacokinetic ramifications for intravitreal drugs, intraocular gene therapy vectors, and possibly vitreoretinal tamponade agents (e.g., silicone oil).

In summary, insights from ophthalmic autopsy and the pathological state of eyes in patients with GD may shed light on the dynamic processes involved in the trafficking of intraocular glucocerebroside. Understanding these processes may ultimately provide insights for new pharmacologic interventions.

Conflict of Interest The authors have no conflicts of interest to report.

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Genetic Background of Uveitis in Chinese Population

30

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Abstract

Uveitis, known as inflammation of the uvea consisting of the iris, ciliary body, and choroid, is one of the leading causes of blindness in the world. Behcet's disease (BD), Vogt-Koyanagi-Harada (VKH) syndrome, and acute anterior uveitis (AAU) are three commonly seen uveitis entities in China. Although the precise pathogenesis remains unclear, accumulating evidence shows that complex genetic backgrounds may be implicated in the development of uveitis. Genes encoding for human leukocyte antigens (HLAs) have been shown to be associated with these uveitis entities, including BD (HLA-B51), VKH syndrome (HLA-DR4, DRB1/DQA1), and AAU (HLA-B27). Genome-wide association studies showed that the IL23R locus was a shared risk factor for these three uveitis entities including BD, VKH syndrome, and AAU. In addition, various other non-HLA genes are also associated with BD, VKH syndrome, or AAU, such as IL-10, STAT4, miR-146a, miR-182, and FoxO1. Moreover, copy number variants (CNV) of complement component 4 (C4), IL17F, IL23A, FoxP3, FAS, and C5 have been found to be associated with BD, VKH syndrome, or AAU. In conclusion, these studies support the hypothesis that genetic factors play a key role in the pathogenesis of these uveitis entities.

Keywords

Uveitis • BD • VKH syndrome • AAU • Genetic factors

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30.1 Introduction

Uveitis, known as inflammation of the uvea consisting of the iris, ciliary body, and choroid, is one of the leading causes of blindness in the world. It is estimated that uveitis accounts for 10–15% of the blindness in the Western world [1]. There are 3–5 million patients with uveitis in China based on a conservative estimation. It is important to note that the blindness caused by uveitis is usually permanent and irreversible because the retina and optic nerve are damaged by inflammation.

Uveitis can be caused by infectious and non-infectious mechanisms, whereby the latter entities are often associated with extraocular involvement. Behcet's disease (BD) is a chronic multisystemic inflammatory disorder characterized by recurrent uveitis, skin lesions, and oral and genital mucous ulcers. This disease is highly prevalent along the ancient Silk Road [2], involving many countries from Asia to the Mediterranean basin, such as China, Korea, Japan, and Turkey. Vogt-Koyanagi-Harada (VKH) syndrome is an autoimmune disease characterized by a bilateral granulomatous panuveitis, poliosis, vitiligo, alopecia, and central nervous system and auditory signs. VKH syndrome mainly affects some pigmented races, such as Asians and Native Americans [3, 4]. Acute anterior uveitis (AAU) is the most common form of uveitis, being characterized by sudden onset, self-limiting, and recurrent inflammation involving the iris and ciliary body [5]. The development of AAU may result in vision loss secondary to complicated cataract and glaucoma [6]. AAU is also considered to be the most frequent extra-articular feature in ankylosing spondylitis (AS) and can affect up to one third of patients with spondyloarthritis [7–9].

Although the etiology and pathogenesis of BD, VKH syndrome, and AAU is not completely clear, the imbalance of pathogenic Th1/Th17 and regulatory T cells is considered to be involved in the pathogenesis of uveitis, and accumulating evidence also shows that complex genetic backgrounds may be involved in the development of uveitis. Human leukocyte antigen (HLA) genes have been shown to be associated with BD (HLA-B51), VKH syndrome (HLA-DR4, DRB1/DQA1), and AAU (HLA-B27). Recently, genome-wide association studies (GWAS) from Japan, Turkey, and China have shown that not only genes of the HLA system predispose to uveitis but that variants of many non-HLA genes such as IL-10, IL-23R/IL-12RB2, STAT4 genes, and BD [10–12] are also involved, thereby supporting the hypothesis that genetic factors contribute to the pathogenesis of this disease. GWAS also showed that the IL-23R gene was associated with VKH syndrome in a Chinese Han population [13]. Additionally, the IL-23R gene was also shown to be associated with AAU [14]. Other genetic variants and copy number variants also showed an association with BD, VKH syndrome, and AAU. The aim of this chapter is to provide further detail concerning the pathogenesis of uveitis entities such as BD, VKH syndrome, and AAU in Chinese Han with an emphasis on new insights in the genetic background (Table 30.1, 30.2, and 30.3).

30.2 HLA Genes Associated with Uveitis

HLA molecules play an important role in antigen presentation, and their genetic diversities are thought to play a dominant role in species survival from microbial infection [15–17]. HLA genes have been demonstrated to be significantly associated with many types of uveitis in different regions and races. For example, HLA-B51 was proven to be strongly associated with BD in different ethnic groups [10, 11, 18–20]. In addition, some genes within the HLA region such as MICA also suggested a strong association with BD [21, 22]. Besides, HLA-DR4, HLA-DR53, and DRB1/DQA1 were shown to be strongly associated with VKH syndrome in a variety of ethnic groups [13, 23–25]. Moreover, HLA-B27

Table 30.1 Summary of the genes involved in Behcet's disease in China

Genes	Odds ratio	95% confidence interval	Ethnic	References
IL23R	1.86	1.39–2.49	Chinese	[43]
MCP1	1.51	1.05–2.17	Chinese	[56]
SUMO4	1.7	1.3–2.2	Chinese	[35]
FCRL3	0.7	0.5–0.9	Chinese	[88]
CD40	1.98	1.38–2.83	Chinese	[49]
STAT3	1.71	1.24–2.37	Chinese	[53]
JAK1	1.33	1.16–1.51	Chinese	[55]
UBAC2	1.5	1.2–1.7	Chinese	[36]
CCR1/ CCR3	0.28	0.2–0.4	Chinese	[38]
PDGFRL	0.59	0.49–0.72	Chinese	[89]
MIF	1.46	1.19–1.79	Chinese	[91]
TNFAIP3	2.03	1.65–2.49	Chinese	[68]
DHCR7	1.51	1.25–1.82	Chinese	[90]
TLR-2	1.46	1.22–1.75	Chinese	[66]
STAT4	1.45	1.3–1.6	Chinese	[32]
TRAF5	1.58	1.25–2.01	Chinese	[50]
IL12B	1.84	1.39–2.43	Chinese	[40]
miR-146a	1.23	0.95–1.58	Chinese	[75]
miR-196a2	1.63	1.19–2.23	Chinese	[76]
miR-182	0.58	0.46–0.71	Chinese	[64]
FAS	2.16	1.62–2.85	Chinese	[87]
IL10	1.63	1.42–1.87	Chinese	[72]
Rorc	3.0	2.0–4.6	Chinese	[65]
Foxp3	3.1	1.8–5.2	Chinese	[75]

was found to be significantly associated with AAU [9, 26–30].

Table 30.2 Summary of the genes associated with VKH syndrome in China

Genes	Odds ratio	95% confidence interval	Ethnic group	References
STAT4	1.78	1.13–2.81	Chinese	[32]
TRAF5	2.34	1.83–3.00	Chinese	[50]
IL12B	1.39	1.12–1.72	Chinese	[40]
CTLA4	0.76	0.63–0.92	Chinese	[39]
IL-17F	1.52	1.13–2.06	Chinese	[57]
TNFAIP3	1.6	1.3–1.9	Chinese	[69]
JAK1	0.71	0.61–0.83	Chinese	[54]
FGFR1OP	1.25	1.00–1.55	Chinese	[92]
MIF	1.57	1.23–2.00	Chinese	[93]
TNIP1	1.93	1.44–2.60	Chinese	[70]
PTPN22	1.54	1.32–1.80	Chinese	[94]
OPN	1.83	1.20–2.79	Chinese	[52]
miR-182	0.57	0.46–0.70	Chinese	[64]
FAS	2.01	1.55–2.62	Chinese	[87]
CLEC16A	0.6	0.5–0.7	Chinese	[95]
IL23A	3.4	2.8–4.1	Chinese	[86]

Table 30.3 Summary of the genes associated with AAU in China

Genes	Odd ratio	95% confidence interval	Ethnic group	References
TRAF5	0.28	0.19–0.43	Chinese	[51]
TNFSF15	0.60	0.5–0.8	Chinese	[97]
FoxO1	1.86	1.37–2.52	Chinese	[63]
CFI	0.68	0.54–0.88	Chinese	[98]
C5	1.89	1.02–2.35	Chinese	[96]

30.3 Genetic Variations Involved in Th1 Cell Pathways

Previous studies showed that Th1 cells play an important role in the development of uveitis, and an increased expression of transcription factors T-bet and IFN-γ is found in uveitis patients [31]. Multiple association studies of candidate genes involving Th1 cell pathways were performed in Chinese Han. Signal transducer and activator of transcription protein 4 (STAT4) is a critical factor for Th1 development. Several SNPs at STAT4 including rs897200, rs7574070, and rs7572482 were found to be associated with BD in a Chinese Han population [12]. Further functional studies showed that the rs897200 risk genotype in STAT4 has a pathogenic role by upregulating the STAT4 transcription and IL-17 production [12]. Hu et al. [32] showed that the TT genotype of STAT4/rs7574865 may be a susceptibility locus for VKH syndrome in Chinese Han, whereas the GG genotype of this SNP may confer susceptibility for uveitis in male BD patients.

Previous studies suggested that ubiquitination reactions are involved in the regulation of receptor tyrosine kinase signaling and that they may play important roles in the TNF-α, IL-1β, and TCR-mediated NF-κB activation pathway

[33, 34]. Hou et al. [35] identified a polymorphism in a ubiquitin-related gene, SUMO4, that was associated with the susceptibility to BD in a Chinese Han population. Furthermore, Hou et al. [36] confirmed UBASH3B and ubiquitin-associated domain containing 2 (UBAC2) as susceptibility genes for BD in Chinese Han, and further functional study showed that the risk T allele of rs3825427 in UBAC2 was associated with a decreased promoter activity and mRNA expression.

Previous study demonstrated that the C-C chemokine receptor type 1 (CCR1) and CCR3 were upregulated by INF-γ [37], which is the hallmark cytokine of Th1 cells. A recent study identified three SNPs rs13084057, rs13092160, and rs13075270 in CCR1/CCR3 gene to be associated with BD in Chinese Han [38]. Functional studies showed that carriers with the protective allele of rs13092160 have a higher expression of CCR1 and an increased migration of monocytes [38], indicating that CCR1/CCR3 was a novel locus involved in the pathogenesis of BD.

Cytotoxic T lymphocyte antigen 4 (CTLA-4) is a transmembrane receptor on T cells, which transfers an inhibitory signal to T cells, resulting in a decreased IL-2 production. Recently, Du et al. [39] investigated the association of CTLA-4 gene polymorphisms with BD and VKH syndrome in a Chinese Han population. The results showed that the frequencies of both the G allele at the +49 site and haplotype -1661A:-318C:+49G:CT60G were significantly higher in patients with VKH syndrome than that in controls, suggesting that CTLA-4 confers risk to VKH syndrome [39].

Interleukins are important cytokines that can play a role in the development of uveitis. Li et al. [40] investigated the associations of IL12B, IL-12Rbeta1, and IL-12Rbeta2 with BD and VKH syndrome in Chinese Han. The result showed that IL-12B/rs3212227 was involved both in the susceptibility to BD and VKH syndrome, whereas no significant associations were found for IL-12Rbeta1 and IL-12Rbeta2 either in BD or VKH syndrome.

30.4 Genetic Variations Involved in Th17 Cell Pathways

Recently, Th17 cells (IL23/IL17 signaling pathway) were identified to play an essential role in the development of BD and VKH syndrome [41, 42], indicating that the genes related to the Th17 cell pathways may be involved in the pathogenesis of these diseases.

Jiang et al. [43] showed that rs17375018 and rs11209032 in the IL23R gene conferred susceptibility to BD in a Chinese Han population. Several other studies confirmed the association of IL23R with BD in different ethnic populations including Japanese, Turkish, and Iranian patients [10, 11, 44]. Most recently, Hou et al. [13] performed a GWAS in a group of 1538 patients with VKH syndrome and 5603 healthy controls and showed a significant association between IL23R-C1ORF141 and ADO/ZNF365/EGR2 with VKH syndrome. All the five non-HLA genes were expressed in the human iris tissue. IL23R was expressed in the ciliary body, whereas EGR2 was expressed in the choroid and ciliary body. The risk G allele of IL23R/rs117633859 showed low transcriptional activation and was associated with decreased mRNA expression of the IL23R gene [5].

CD40, a member of the TNF receptor family, is expressed on antigen-presenting cells (APCs) and produces the second signal of cell activation through interaction with its ligand (CD40L) [45]. This interaction has been shown to be associated with a number of inflammatory and autoimmune diseases [46–48]. Chen et al. [49] showed that the TT genotype of rs4810485 and rs1883832 of CD40 were significantly associated with BD but not with VKH syndrome in a Chinese Han population. The interaction between CD40 and CD40L is regulated by tumor necrosis factor receptor-associated factor (TRAF), and recent studies suggested that rs6540679, rs12569232, and rs1086388 in TRAF5 as well as rs13210247 in TRAF3IP2 were associated with BD and VKH syndrome [50]. Furthermore, an increased expression of TRAF5 and an increased production of IL-6 and TNF-α were

observed in carriers with the risk genotype of TRAF5/rs6540679 [50]. Moreover, rs12569232 of TRAF5 was found to predispose to AAU and AS in Chinese Han [51].

Osteopontin (OPN) is a pro-inflammatory cytokine involved in chronic inflammatory diseases, including uveitis by promoting the responses of Th17 cells. Chu et al. [52] found that the OPN serum levels were significantly higher in patients with VKH syndrome than that in healthy controls, and a significantly increased frequency of the OPN rs4754 TT genotype was observed in patients with VKH syndrome compared with healthy controls, suggesting that OPN confers risk to VKH syndrome in Chinese Han. The GG genotype of rs2293152 in STAT3, which is involved in Th17 cell function, has also been shown as a risk factor for BD rather than VKH syndrome [53, 54]. Additionally, JAK1 and MCP-1 genes were also shown to be associated with BD in a Chinese Han population [55, 56], and JAK1 affected the susceptibility to VKH syndrome [54]. Moreover, Shu et al. [57] found that a significantly decreased frequency of the IL-17F/rs763780 C allele and an increased frequency of TT genotype were observed in patients with VKH syndrome compared with healthy controls, indicating that the IL-17F gene may be associated with protection and susceptibility to VKH syndrome. The studies mentioned above suggest that genetic variants associated with Th17 pathways may play an important role in the development of uveitis.

30.5 Genetic Variations Involved in Regulatory T (Treg) Cell and Dendritic Cell (DC) Function

Treg cells are a subpopulation of T cells which modulate the immune system and maintain tolerance to self-antigens, thereby controlling the occurrence of overt autoimmune disease. Decreased percentages of Treg cells in $CD4^+$ T cells were found in patients with BD, VKH syndrome, and AAU compared with controls [58–60]. Recent studies have shown that the transcription factor forkhead box O1 (FoxO1) controls Treg cell development and function [61, 62]. Yu et al. [63, 64] examined the association of the FoxO1gene with BD, VKH syndrome, and AAU, and the results showed that significantly increased frequencies of the FoxO1/rs2297626 AA genotype and A allele contribute to the genetic susceptibility of AAU with AS, whereas no association between FoxO1 and rs2297626 was observed with BD and VKH syndrome in a Chinese Han population. IL2RA (CD25) and FoxP3 also play a vital role in the regulation of Treg specialization and stability. However, recent studies did not detect an association of IL2RA and FoxP3 gene polymorphisms with BD, VKH syndrome, or AAU [63–65].

DCs are professional antigen-presenting cell (APC) connecting the innate and adaptive immune responses. The Toll-like receptor (TLR) is an important pattern recognition receptor, which is widely expressed on the surface of DCs. Fang et al. [66] investigated the association of TLR2, TLR4, TLR8, and TLR9 with BD, VKH syndrome, and AAU, and the results showed that the TLR2 gene was involved in the susceptibility to BD rather than VKH syndrome or AAU, whereas TLR4, TLR8, and TLR9 were not found to be associated with uveitis entities such as BD, VKH syndrome, and AAU. Recent studies have shown that the NF-κB pathway plays a vital role in DC development and function, whereby the ubiquitin-modifying enzyme A20 has been shown to play a role in the activation of this pathway [67]. Li et al. [68] found a significantly increased prevalence of the rs9494885 TC genotype and C allele of the A20-encoding gene TNFAIP3 in BD patients compared with controls. Rs10499194 as well as rs7753873 of TNFAIP3 were significantly associated with BD in a Chinese Han population. Further, TNFAIP3 studies by Li et al. [69] showed that the rs9494885 TC genotype and C allele may be predisposing factors to VKH syndrome, whereas the rs9494885 TT genotype and T allele may provide protection against VKH syndrome. Moreover, Shi et al. [70] investigated the association of TNFAIP3 interacting with protein 1 (TNIP1) with BD and VKH syndrome and

showed that a TNIP1 polymorphism was significantly associated with VKH syndrome but not with BD in Chinese Han. DCs may be involved in the occurrence and development of BD and VKH syndrome by secreting cytokines, and IL10 has been identified as a negative regulator. Recently, rs1800871 and rs1518111 of IL10 were found to confer susceptibility to BD in Chinese Han [71, 72].

30.6 Other Genetic Variants Associated with Uveitis

30.6.1 MicroRNAs

MicroRNAs (miRNAs) are small noncoding RNAs that mediate RNA silencing and have been recently recognized as an important regulator in immune homeostasis. Abnormalities in the miRNA-mediated regulation of immune cell development and function are involved in autoimmunity and inflammatory diseases. A recent study showed that miR-155 expression was significantly decreased in BD patients compared to healthy controls [73], suggesting that miRNAs may play an important role in the development of uveitis. MiR-146a was identified as a negative regulator of innate immunity in systemic lupus erythematosus (SLE) [74]. Zhou et al. [75] investigated the association of miR-146a polymorphisms with BD and VKH syndrome and showed that miR-146a/rs2910164 was associated with BD but not with VKH syndrome in a Chinese Han population. Qi et al. [76] investigated the association of miR-196a2/rs11614913, miR-499/rs3746444, and miR-149/rs2292832 with the susceptibility to BD, VKH syndrome, and AAU and showed significantly increased frequencies of the miR-196a2/rs11614913 TT genotype and T allele in BD, but not in VKH syndrome or AAU. Functional experiments showed a decreased miR-196a expression, an increased Bach1 expression, and an increased production of IL-1beta and MCP-1 in TT cases compared to CC cases. Moreover, Yu et al. [64] observed significantly decreased frequencies of the miR-182/rs76481776 CC genotype and C allele in BD and VKH syndrome in a Chinese Han population. Functional experiments showed a significantly increased expression of miR-182 in TT/CT cases compared to CC cases in anti-CD3/CD28 antibody-stimulated $CD4^+$ T cells. However, no significant association of miR-182/rs76481776 with AAU was observed [63].

30.6.2 Copy Number Variations

Copy number variations (CNVs), known as a source of genetic diversity being as significant as SNPs, include gene duplications and gene deletions. CNVs have been reported to confer susceptibility to several autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, and psoriasis [77–80]. A recent study suggested that the total serum complement C4 level was significantly increased in patients with active BD as compared with controls, but not in AAU patients [81]. Hou et al. [81] performed copy number variation analysis of C4 isotypes (C4A and C4B) and showed a significant association of having more than 2 copies of C4A with BD. Functional assays showed that a high copy number of C4 was positively associated with the expression of C4A and the production of interleukin-6 (IL-6) [81]. Hou et al. [82] also showed that the expression of total C4 in serum was significantly decreased in patients with VKH syndrome as compared with healthy controls. CNV analysis in VKH showed a significantly decreased frequency of more than two copies of C4A or more than four copies of total C4. The different role of C4 in BD versus VKH syndrome may be due to the fact that BD is considered an autoinflammatory disease mediated by an aberrant response against microbial antigens, whereas VKH syndrome is an autoimmune disease directed against melanocyte antigens [83, 84].

Xu et al. [85] also observed that the frequency of having more than two copies of C3 was significantly increased in BD and VKH, whereas CNV of C5 was only associated with BD. mRNA expression in the high CNV group and GG cases

of C3 and C5 was significantly higher compared to other genotypes. Hou et al. [86] found that increased frequencies of having more than two copies of IL17F and IL23A were associated with BD or VKH syndrome, whereas no association of CNVs in IL17A and IL23R was found for BD and VKH syndrome. IL-17F protein levels were positively related with gene copy numbers of IL17F.

Yu et al. [87] found that a high copy number (>2) of FAS was associated with BD or VKH syndrome. A significant upregulated mRNA expression of FAS was observed in anti-CD3/CD28 antibodies-stimulated $CD4^+$ T cells from individuals carrying a high gene copy number (>2) as compared to normal diploid 2 copy number carriers, which is consistent with the significantly higher mRNA expression of FAS both in active patients with BD and VKH syndrome as compared to that in controls. Liao et al. [65] showed that high Rorc CNV was associated with the susceptibility to BD, and a low Foxp3 CNV predisposed to BD in female patients. Further functional studies demonstrated that the relative mRNA expression levels of Rorc were increased in individuals with a high Rorc gene copy number, but not for Foxp3. An increased production of IL-1β and IL-6 was found in individuals carrying a high CNV of Rorc [65]. The studies mentioned above all suggest that CNVs may play an important role in the development of uveitis.

30.6.3 Other Genetic Variants Associated with Uveitis

Recent studies showed that several other loci that are more or less also involved in the immune response, such as FCRL3 [88], PDGFRL [89], DHCR7 [90], and MIF [91], were associated with BD in Chinese Han (Table 30.1). In addition, gene polymorphisms of FGFR1OP [92], MIF [93], PTPN22 [94], and CLEC16A [95] were identified to be associated with VKH syndrome in a Chinese Han population (Table 30.2). Moreover, recent association studies suggested that C5 [96] conferred risk to AAU, whereas TNFSF15 [97] and CFI [98] conferred protection to AAU in Chinese Han (Table 30.3).

30.7 Summary

With the continuous progress of unraveling the genetic background of individuals suffering from uveitis, more and more genetic susceptibility factors associated with uveitis have been identified. These studies have markedly increased our knowledge concerning the immunological and inflammatory pathways involved in the pathogenesis of several uveitis entities such as BD, VKH syndrome, and AAU. However, many pathogenic genes for uveitis still need to be identified, as well as their interaction with environmental factors. Differences between ethnic groups and geographical regions necessitate a further validation of the candidate pathogenic genes in different populations throughout the world. In addition, the detailed pathogenic mechanism of candidate genes needs to be elucidated at both the RNA and protein level. A greater understanding of the genetic pathogenic mechanisms underlying BD, VKH syndrome, and AAU may provide a foundation for developing new strategies to improve the diagnosis and treatment of uveitis.

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Usher Syndrome in Chinese and Japanese Population

31

Shi-Ying Li, Linghui Qu, Xiaohong Meng, and Zheng Qin Yin

Abstract

Usher syndrome (USH) is the most common cause of combined blindness and deafness inherited in an autosomal recessive mode. It is clinically and genetically heterogeneous and is the most common cause underlying deafness and blindness of genetic origin. Molecular diagnosis is of great significance in revealing the molecular pathogenesis and aiding the clinical diagnosis of this disease. Recent molecular findings have provided more information regarding the pathogenesis of this disorder and the wide phenotypic variation in both audio vestibular and visual systems. However, molecular diagnosis remains a challenge due to high phenotypic and genetic heterogeneity in USH. Many results revealed a different prevalence of causative genes among different ethnicities, which highlight the importance of making specific genetic catalogues for each ethnicity. This chapter catalogued the comprehensive molecular diagnosis of a large cohort of Chinese and Japanese USH population.

Keywords

Hearing loss; Next-generation sequencing; Retinitis pigmentosa; Usher syndrome

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31.1 Introduction

Usher syndrome (USH) is an autosomal recessive disorder with a prevalence of 3.2–6.2/100,000 worldwide that is characterized by the association of sensorineural hearing loss (HL), visual impairment due to retinitis pigmentosa (RP), and variable vestibular dysfunction and exhibits remarkable clinical and genetic heterogeneity [1, 2]. It affects about one child out of 25,000 and accounts for >50% of patients who are both deaf and blind [2] around the world.

Visual acuity loss due to typical RP, congenital hearing impairment, and variable vestibular dysfunction are the primary clinical symptoms. Three clinical subtypes of USH (USH1, USH2, and USH3) are distinguished depending on the severity and progression of HL and the presence or absence of vestibular areflexia. These distinctions are generally used to guide molecular diagnosis. USH1 is the most severe form and is characterized by prepubertal onset RP, profound HL, and vestibular dysfunction. USH2 is the most common clinical form of the disorder, accounts for over half of USH cases, and is characterized by postpubertal onset RP and moderate-to-severe HL without vestibular dysfunction. USH3 is characterized by postlingual HL, teenage-onset RP, and varying degrees of vestibular dysfunction [3]. However, some cases are not easily classifiable under these categories and could be categorized as atypical USH [4].

To date, mutations in 12 genes have been implicated in the etiology of USH, and 3 additional loci (USH1E, USH1H, and USH1K) have been identified so far [5]. Six causative genes have been reported for USH1: *MYO7A* (USH1B), *USH1C* (USH1C), *CDH23* (USH1D), *PCDH15* (USH1F), *USH1G* (USH1G), and *CIB2* (USH1J) [6]. Mutations in *MYO7A* are the most prevalent [7, 8]. Three genes have been identified as associated with USH2: *USH2A* (USH2A), *GPR98* (USH2C), and *DFNB31* (WHRN) (USH2D). *USH2A* mutations are the most common of this group [7, 8]. Mutations in *CLRN1* (USH3A) have been pinpointed as a cause of USH3 [9], and the HARS gene was recently proposed as a putative USH3 gene [10]. Additionally, a 12th gene, *PDZD7*, has been described as a contributor to digenic USH and as a modifier of the retinal phenotype [11]. It is also worth noting that mutations in many of the USH genes can also lead to deafness without a retinal phenotype [5, 12]. In total, these genes comprise 337 coding exons that span a length of 62,420 nucleotides (Table 31.1).

The spectrum of disease genes is not quite similar between Asian patient cohort and other patient cohorts from different (and primarily Caucasian) ethnic backgrounds. To date, quite a few studies have been reported on the molecular diagnoses of Asian USH patients. In order to gain a better understanding of the molecular and genetic etiology in Chinese and Japanese

Table 31.1 12 genes implicated in the etiology of Usher syndrome

No.	Gene	Location	Exon no.	Length (bp)	Amino acid
1	MYO7A	11q13.5	48	6648	2215
2	USH1C	11p14.3	27	2700	899
3	CDH23	10q22.3	22	3345	1114
4	PCDH15	10q21.1	32	5868	1955
5	USH1G	17q25.1	3	1386	461
6	CIB2	15q25.1	5	435	144
7	USH2A	1q41	72	15,609	5202
8	GPR98	5q13	90	18,921	6306
9	DFNB31	9q32	12	2724	907
10	CLRN1	3q25	3	699	232
11	HARS	5q31.3	13	1530	509
12	PDZD7	10q24.31	10	1555	517
Total			**338**	**62,420**	**20,461**

ethnicity, we catalogued the comprehensive molecular diagnosis of a large cohort of Chinese and Japanese Usher syndrome patients associated with their diverse disease phenotypes.

31.2 Methods

31.2.1 Methods of Molecular Diagnosis

Molecular genetic diagnosis for USH has developed from the scanning of restricted portions of USH genes to extensive direct sequencing. Because of the genetic heterogeneity, prioritization of the genes to be sequenced was achieved by preliminary linkage analysis. Due to the large size of most USH genes (in total more than 350 exons), Sanger sequencing of genes one by one remains expensive and time-consuming. Furthermore, large rearrangements have been described in *MYO7A*, *CDH23*, *GPR98*, *USH2A*, and particularly *PCDH15*, and their detection requires array CGH studies and/or multiplex ligation-dependent probe amplification. Taken together, these strategies allow a reliable diagnosis for Usher patients with a mutation detection rate of about 90% for USH1 and USH2 patients. A genotyping microarray commercially available allows rapid screening for hundreds of previously identified variations in USH genes, but its application in clinical diagnosis is hampered by a very low detection rate as most USH causing DNA alterations are private or restricted to one or two families [3].

Next-generation sequencing (NGS) technology has recently demonstrated its capacity to detect DNA variants in sensorineural disorders known to be genetically heterogeneous. Targeted exome sequencing (TES) based on NGS has been proven to be a powerful, robust, precise, and cost-effective tool for discovering genetic mutations in large genomic regions [3, 13–18]; targeted NGS is an accurate and effective method for detecting genetic mutations related to USH. The capture panel consisted of base pairs (bp) covering all coding and noncoding exons with flanking exon/intron boundaries (±10–25 bp) of retinal and optical disease genes and genes expressed at the highest level in rod or cone photoreceptors. Whole-exome sequencing (WES) is a procedure that allows the purification by sequence capture of all exonic regions of a genome and their further processing by NGS [13].

31.2.2 Determination of the Pathogenicity of the Variants [19]

A variant was classified as pathogenic if the following criteria are applied. (1) Variants were excluded that had an allele frequency >0.5% (for recessive variants) or >0.1% (for dominant variants) in any of the 1000 Genomes database, ESP6500 database, or HGVD. (2) Mutations listed in the HGMD or those identified as pathogenic alterations in previous publications were regarded as pathogenic. (3) Nonsense and frameshift variants were also considered as pathogenic. (4) For a novel missense variant, in silico prediction programs were used to predict its pathogenicity. Only novel missense variants that were predicted to be pathogenic by at least five of seven well-established algorithms were reported. Variants that were predicted to be pathogenic by at least three of five missense prediction programs (SIFT, Polyphen2, LRT, MutationTaster, and Mutation-Assessor) and whose evolutionary conservation scores were >0 in both PhyloP and GERPtt were considered to be pathogenic. (5) For splice site variants, the prediction program MaxEntScan was used, and these were considered pathogenic if the score differed by >5 between the wild-type and mutated sequences. (6) Variants were adopted that matched the patients' phenotype and the reported inheritance pattern of the respective genes. Pathogenic variants in the dominant genes found in simplex cases were regarded as disease-causing mutations only when they were published previously or were confirmed to be a de novo mutation by using parental testing. Variants with a frequency more than that mentioned in criterion 1 were excluded even if they were listed in the HGMD or in previously published reports. All mutations and potential pathogenic variants

detected using NGS were validated using conventional Sanger sequencing. Sequencing was performed using an Applied Biosystems (ABI) 3130xl Genetic Analyzer (Life Technologies). Segregation analysis was performed if DNA from family members was available.

31.3 Genetic Findings of Japanese Patients

31.3.1 Japanese USH1 Patients

Hiroshi Nakanishi et al. [20] described the first mutation analysis of *MYO7A* and *CDH23* in five Japanese USH1 patients in 2010 with polymerase chain reaction (PCR) and Sanger sequencing technology. Five mutations (three in *MYO7A*, p.Arg150X, p.Ala771Ser, and p.Arg1883Gln; two in *CDH23*, p.Tyr1942SerfsX23 and p.Arg2107X) were identified in four of five unrelated patients. Of these mutations, two mutations (p.Tyr1942SerfsX23 in *CDH23* and p.Ala771Ser in *MYO7A*) were novel at that time.

Hidekane Yoshimura et al. [21] had conducted genetic analysis to find mutations in nine causative USH genes (except *CIB2*) in Japanese USH1 patients, using targeted exon sequencing of selected gene technology combined with direct sequence analysis technique. They screened 17 unrelated Usher syndrome type 1 patients and detected probable pathogenic variants in 16 of them (94.1%) who carried at least one mutation. Seven had *MYO7A* mutations (41.2%), three had *CDH23* mutations (17.6%), and two had *PCDH15* mutations (11.8%). Four USH1 patients had probable pathogenic mutations in two different USH genes, with one being a biallelic mutation. The other heterozygous/homozygous mutations were missense variants. Three of these patients presented with earlier RP onset (night blindness) than in the other patients with two pathogenic mutations ($p = 0.007$). One patient had heterozygous mutations in two USH1 genes (p.Ala771Ser in *MYO7A* and c.158-1G>A in *PCDH15*).

Using targeted exon sequencing of selected genes with massively parallel DNA sequencing (MPS) technology, Hidekane Yoshimura et al. [22] screened 227 unrelated non-syndromic deaf children and detected recessive mutations in USH1 causative genes in five patients (2.2%): three patients harbored *MYO7A* mutations and one each carried *CDH23* homozygous mutations (c. 2090–2093 insG, P.L. 697 fs) or *PCDH15* mutations (c.289C>T, p.Q97X; c.334C>T, p.R112X). As indicated by an earlier genotype–phenotype correlation study of the *CDH23* and *PCDH15* genes, they considered the latter two patients to have USH1. This first report describing the frequency (1.3–2.2%) of USH1 among non-syndromic deaf children highlighted the importance of comprehensive genetic testing for early disease diagnosis.

31.3.2 Japanese USH2 Patients

Hiroshi Nakanishi et al. [23] first reported the results of scanning for USH2A mutations in Japanese patients with USH2 in 2009. In 8 of 10 unrelated patients, they identified 14 different mutations. Of these mutations, 11 were novel at that time. p.Glu767fs, the most prevalent mutation in European Caucasians that accounted for approximately 30% of mutated alleles, was not detected in their study. p.Thr3571Met was the only mutation that was common between Japanese and other populations, including European, French Canadian, Jewish, and Palestinian. The possible frequent mutation c.8559-2A>G was identified in four of ten patients and accounted for 26.7% of mutated alleles; it was thus a frequent mutation in Japanese patients. Hence, they believed mutation screening for c.8559-2A>G in *USH2A* might prove very effective and practicable for the early diagnosis of Japanese USH2 patients.

To obtain a more precise mutation spectrum, they analyzed further nine Japanese patients in another study [24]. They identified nine mutations, of which eight were novel at that time. The result indicated that the mutation spectrum for *USH2A* among Japanese patients largely differs from Caucasian, Jewish, and Palestinian patients. The p.Arg1777Trp missense mutation

was identified in two patients, whereas the other mutations were detected in one patient each. Meanwhile, they did not find the c.8559-2A4G in that study. Haplotype analysis of the c.8559-2G (mutated) alleles using 23 single nucleotide polymorphisms surrounding the mutation revealed an identical haplotype pattern of at least 635 kb in length, strongly suggesting that the mutation originated from a common ancestor. The fact that all patients carrying c.8559-2A4G came from western Japan suggested that the mutation was mainly distributed in that area.

31.3.3 Japanese USH3 Patients

Hidekane Yoshimura et al. [25] examined the *CLRN1* gene mutation analysis in Japanese patients who were diagnosed with Usher syndrome type 3 (USH3) on the basis of clinical findings and identified the novel homozygous pathogenic mutation c. 606T>G (p. N202K) in the *CLRN1* gene in two patients. This was the first report of USH3 with a *CLRN1* gene mutation in Asian populations.

31.4 Genetic Findings of Chinese Patients

31.4.1 Chinese USH1 Patients

Fei Liu et al. [26] identified two novel mutations of *MYO7A* in a Chinese non-consanguineous family with USH1 with direct DNA sequencing of *MYO7A*: c.3742G>A (p.E1248K) and c.6051 +1G>A (donor splicing cite mutation in intron 44). The c.3742G>A mutation was predicted to cause a glutamic acid to lysine change at codon 1248. The c.6051+1G>A was a G to A change at the conserved donor splice site in intron 44. Based on severe hearing impairment, unintelligible speech, and retinitis pigmentosa, the family proband and the affected individual were clinically diagnosed with Usher syndrome type 1.

Weining Rong et al. [27] identified three new alleles and one known mutation in *MYO7A* gene using targeted NGS approach in the three families in 2014. In two families with USH type 1, novel homozygous frameshift variant p. Pro194Hisfs*13 and recurrent missense variant p.Thr165Met were demonstrated as the causative mutations, respectively.

Qu et al. [28] identified three novel mutations, compound heterozygous mutations c.4398G>A and EX38-49del in *MYO7A* and a homozygous mutation c.988_989delAT in *USH1C*, in Chinese USH1 families based on targeted next-generation sequencing (NGS) technology. The mutation c.4398G>A led an amino acid substitution of a termination codon for tryptophan at codon 1466 (p.Trp1466Ter), which caused premature termination of the amino acid coding of the *MYO7A* gene. The mutation EX38-49del was large fragment deletions in exons 38–49 (EX38-49del: 1480 bp deletion) in *MYO7A*. Mutation c.988_989delAT in *USH1C* caused a proline substitution for isoleucine at codon 330 and was expected to lead to a frameshift and premature termination of translation after insertion of 24 irrelevant amino acids starting at the position 330 of proline (p.Ile330ProfsX24). The phenotypes of the two families' patients with these mutations were very severe, which is consistent with the predicted mutant allele severities. The probands exhibited a delay in gross motor development and profound HL from birth due to USH1. However, one of the probands was misdiagnosed with neonatal hypoxic-ischemic encephalopathy shortly after birth. Molecular genetic diagnosis has great potential to reveal the molecular pathogenesis, aid the early and differential diagnosis, and explain the severe clinically observed symptoms of this disease.

Lichun Jiang et al. [5] detected mutations in 10 out of 14 Chinese USH1 patients in 2015. Consistent with previous reports, *MYO7A* was the most frequently mutated gene with 13 different pathogenic mutations found in eight patients. Among them, three have been previously reported as pathogenic alleles, while the other ten alleles are novel, including three frameshift mutations, two splicing site mutations, three nonsense mutations, and two missense mutations. Additional putatively pathogenic mutations were identified in *PCHD15*

and *CLRN1* in this group of USH1 patients. Patient USHsrf8 was found to carry compound heterozygous mutations in *PCDH15*, consisting of the novel frameshift mutation *PCDH15*: c.1799_1800insTA (p.S600fs) and the novel nonsense mutation *PCDH15*:c. 2893A > T (p.R965X). Interestingly, patient USHsrf14 had an unexpected molecular diagnosis because patient USHsrf14 was diagnosed with USH1 but has mutations in *CLRN1* which have previously been reported to cause mostly USH3. This patient was found to carry novel nonsense mutation *CLRN1*:c. 658C > T (p. R220X) and novel missense mutation *CLRN1*:c. 190G > A (pG64R), which is predicted to be pathogenic.

31.4.2 Chinese USH2 Patients

Xuezhong Liu et al. [29] screened the c.2314delG mutation in five Chinese families with USH2 and atypical USH and detected one homozygous and one heterozygous c.2314delG in 1999. This was the first report to describe mutation analysis of the complete coding sequence of *USH2A* in the Chinese population. The mutation 2314delG lies within a crucial region in the laminin epidermal growth factor motif of *USH2A* and results in a frameshift at codon 772 and a premature stop codon 20 amino acids downstream, presumably leading to a truncated protein and loss of function. The phenotypic variation observed in individuals with the 2314delG mutation suggested that environmental or genetic factors affected expression of *USH2A*.

Hanjun Dai et al. [30] identified five novel *USH2A* mutations in three unrelated Chinese USH2 families with PCR and direct sequence analysis in 2008. Of the five mutations, four were truncating mutations: c.99_100insT (p.R34fs), c.8483delC (p.S2828fs), c.8559–2A>G (Exon43DEL), and c.9450G>A (p.W3150X). The only missense mutation of c.11806A>C (p.T3936P) replaced a polar threonine with a nonpolar hydrophobic proline and was predicted to be pathogenic. Six patients in the three unrelated families showed typical clinical phenotype of USH2.

Wenjun Xu et al. [31] identified six *USH2A* mutations in three unrelated Chinese USH2 families with PCR and direct DNA sequencing in 2011. They also supported that two novel mutations of *USH2A* were responsible for non-syndromic retinitis pigmentosa (RP). Among the eight mutations, seven were novel.

Weining Rong et al. [27] detected novel biallelic heterozygous mutations in *MYO7A*: c. 1343+1G>A and c. 2837 T>G (p.Met946Arg), in the two patients from the family affected with USH type 2. *MYO7A* mutations had rarely been correlated with USH type 2. This study had summarized the clinical findings of a Chinese family with USH type 2, in which they identified novel biallelic *MYO7A* mutations.

Xiufeng Huang et al. [14] utilized TES to study genetic defects in five USH2 families. Eight affected patients and 12 unaffected relatives from 5 unrelated Chinese USH families, including 2 pseudodominant ones, were recruited. Eleven mutations, eight of them were novel, in the *USH2A* gene were identified.

Xue Chen et al. [32] also used targeted NGS technology to reveal *USH2A* mutations associated with diverse disease phenotypes. They detected two *USH2A* heterozygous novel mutations, c.8223+1G>A (P.V2742Ifs*3) and c.11156G>A (P.R3719H), in a USH2 family. They also detected *USH2A* mutations responsible for early-, moderate-, or late-onset non-syndromic retinitis pigmentosa and retinitis pigmentosa sine pigmento.

Qu et al. [28] described six *USH2A* mutations in three USH2 Chinese families using gene panel-based NGS: c.15104_15105delCA (p.Thr5035ArgfsX142) and c.10712C>T (p.Thr3571Met), c.5329C>T (p.Arg1777Trp) and c.6875_6876insG (p.Arg2292ArgfsX39), and c.8559-2A>G and c.9570+1G>A. They identified two novel truncation mutations (c.15104_15105delCA and c.6875_6876insG) in the *USH2A* gene. These two truncation mutations were combined with two different previously reported missense mutations

(c.10712C>T and c.5329C>T, respectively). The mutation c.10712C>T has been detected in USH patients from Europe, including Spain and France [33–35], but this was the first time this mutation had been reported in Asia. The mutation c.5329C>T had been identified in two Japanese patients along with two different truncation mutations (p.Ile3055MetfsX2 and p. Pro2404ValfsX9) [24]. The mutation types in patients of F4 and the two reported Japanese patients were one truncating mutation in combination with the same missense mutation (c.5329C>T), and these patients exhibited similarly moderate phenotypes that included the following: moderate HL, night blindness onset ages of 14–17, and BCVAs better than 0.1 (despite one of the Japanese patients being 58 years old) [24]. However, the phenotypes of the F3 patients were more severe than those of the F4 patients; the F3 patients exhibited more severe HL, earlier onset of night blindness (7–8 years vs. 16–17-years), and worse BCVAs (below 0.1 vs. 0.4 in patients in their thirties). This pattern of findings might partially have resulted from c.10712C>T potentially causing more severe damage to protein function than c.5329C>T, for their allele mutations were both truncating mutations. Further experiments are needed to reveal the relevant functional changes. The compound heterozygous splicing mutations of c.8559-2A>G and c.9570+1G>A have been reported previously in Japanese and Chinese patients [17, 23, 30]. The mutation c.8559-2A>G in *USH2A* has been found to be a frequent mutation among Japanese USH2 patients [23].

Suilian Zheng et al. [36] identified one novel (c.4576G>A, p.G1526R) and two known mutations (c.5581G>A, p. G1861S; c.2802T>G, p. C934W) in the *USH2A* gene in a pseudodominant USH2 family using whole-exome sequencing technology. They demonstrated that whole-exome sequencing is a robust approach for the genomic diagnosis of disorders with high degrees of genomic heterogeneity.

Lichun Jiang et al. [5] identified mutations in 39 of 54 Chinese USH2 and atypical patients applying targeted next-generation sequencing technology. Biallelic mutations were detected in 39 USH2 or USH2-like patients, with *USH2A* mutated in 32 patients, *GRP98* mutated in 3 patients, *CLRN1* mutated in 2 patients, *MYO7A* mutated in 1 patient, and *DFNB31* mutated in 1 patient. Consistent with previous reports, they found that *USH2A* was the most frequently mutated gene in USH2 patients, accounting for about 60% (32 out of 54) of patients in this cohort. A total of 40 different mutations were identified in *USH2A*, including 27 novel alleles. *GPR98* was the second most frequently mutated gene in their USH2 patients, with pathogenic mutations occurring in three patients. Two homozygous mutations in USH type 3 gene *CLRN1* were found in two USH2 patients. Compound heterozygous missense variants in USH type 1 gene *MYO7A* were identified in USH2 patient USHsrf40, who carried two missense variants c.4951G > A (p. D1651N) and c.4360G > A (p.V1454I). Novel homozygous splicing site mutation c.963 + 1G > A in DFNB31 was found in a USH2 patient from a consanguineous family, which was confirmed by segregation tests.

Interestingly, they observed a strong enrichment for severe protein-truncating mutations expected to have severe functional consequence on the protein in USH2 patients compared to the reported mutation spectrum in RP patients, who often carried partial protein-truncating mutations.

Hairong Shu et al. [37] reported a homozygous frameshift mutation c.4382delA (p. T1462Lfs*2) in exon 20 of gene *USH2A* in the Chinese USH2 family using targeted next-generation sequencing technology. Two compound heterozygous mutations, IVS47 + 1G > A and c.13156A > T (p.I4386F), located in intron 48 and exon 63, respectively, of *USH2A*, were identified as causative mutations for the second USH2 family. Of note, the missense mutation c.13156A > T had not been reported so far.

Ji Yang et al. [38] reported two novel compound heterozygous truncation mutations c.6382C>T (p.R2128X) and c.17099_17100delAC (p.N5700Tfs*7) in *GPR98* in a Chinese USH2 family. There are a few reports

of molecular diagnosis in Chinese USH families with truncation *GRP98* mutations, and their results indicated a distinctive mutation spectrum in this population.

31.4.3 Chinese USH3 Patients

There were very little reports about the molecular diagnosis of Chinese USH3 patients until now.

31.5 Diagnosis and Potential Treatment

31.5.1 Diagnosis

Usher syndrome (USH) is a clinically and genetically heterogeneous disorder which is important from a public health viewpoint because of the social isolation which Usher patients must endure. USH is not only a physical disorder but also associated with psychosis, mood, and behavioral disorders [39].

Genetic tests could be a very powerful tool in differential diagnosis of USH patients; the identification of causative mutations is of great significance for revealing the molecular pathogenesis and aiding the clinical diagnosis of this disease, as the recent literatures reported in the Chinese and Japanese population. However, there are many factors that make the genetic study of this disease a complicated, difficult one. The genes identified to date do not explain all the USH cases (this is true for Bardet–Biedl syndrome and Alström syndrome as well), and the variable nature of the proteins involved in USH and the complexity of the USH interactome make identifying novel genes a difficult task. This is due to genetic and allelic heterogeneity, which contribute to the low rate of mutation detection, together with the possible presence of large deletions, mutations in noncoding regions, or isoforms in low concentration only present in the affected tissues. Moreover, other complex inheritance forms could modify the phenotype and its expression, as recently shown by Ebermann et al. All of these factors make the use of traditional techniques for mutation detection difficult. Advances in massive sequencing technologies will certainly change the approaches to molecular diagnosis of Usher syndrome [1]. NGS technology has recently demonstrated its capacity to detect DNA variants in inherited disorders and guarantee a quality of coverage in coding sequences of target genes that is suited for diagnostic purposes; moreover, NGS is thought to be highly efficient and cost-effective [3, 13–18]. Gene characterization and mutation screening will unravel the functional aspects and allow a phenotype–genotype correlation to be established.

The diagnosis could hardly rely on ocular clinical manifestations since it was highly variable. The fERG could be extinguished, decreased, or normal due to different stages of the diseases. OCT displayed thinner retinal thickness and disorganized inner and outer segment structure. AF revealed RPE dysfunction, and fundus color photograph may display bone-spicule hyperpigmentation and attenuated arteries.

31.5.2 Therapy [40]

To date, apart from hearing aids and cochlear implantation designed to correct hearing impairment, there is no “authentic” therapy for USH patients. In addition, these hearing devices are of little benefit for understanding speech in noisy environments, hence the need for developing alternative therapeutic approaches. One of the subtypes, Usher 1B, is caused by loss of function of the gene encoding the unconventional myosin, MYO7A. A variety of different viral-based delivery approaches have been tested for retinal gene therapy to prevent the blindness of Usher 1B. A phase 1 clinical trial has been approved by the Food and Drug Administration (FDA) and has been under way in the USA since 2012, and a second trial has been approved in France.

A variety of viral-based delivery approaches have been tested for Usher 1B retinal gene therapy, and most have been shown to be efficacious. Each has its advantages and disadvantages.

Recent studies on the use of AAV for the delivery of *MYO7A* have been encouraging, especially because of the relatively established success of AAV in gene therapy for inherited retinal diseases such as LCA2. One alternative that may prove to be optimal for large genes like *MYO7A* is the use of nonintegrating lentiviruses; integrase-deficient vectors that can transduce retinal cells are able to persist as episomal DNA. However, such studies have yet to be reported.

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Homozygosity Mapping for Autosomal Recessive Ocular Diseases

32

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Abstract

Genetic eye diseases play a major role in causing visual impairment and blindness affecting all the structures of the eye from the anterior to posterior segment. They are inherited as either Mendelian or complex and in Mendelian inheritance, auotosomal dominant, autosomal recessive or X-linked recessive patterns are commonly observed. Linkage and homozygosity mapping is based on the information provided by the non-recombinants on the chromosomal segments to map disease genes. In Homozygosity mapping the large stretches of homozygous alleles inherited due to identity-by-descent (IBD) and shared between the affected individual(s) and absent in the unaffected in the family are compared to map the disease gene locus. A small family with one each of affected and unaffected provides sufficient data to map disease locus/gene using homozygosity mapping unlike linkage studies where larger multi-generation families with many affected and unaffected are required. An increased prevalence of autosomal recessive disorders observed in genetically isolated or highly inbred families have aided in mapping many ocular diseases genes using homozygosity mapping. This chapter details the history, methodology and the different ocular diseases where homozygosity mapping has been applied to identify the causative genes.

Keywords

Homozygosity mapping • Autosomal recessive • Inherited ocular diseases

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32.1 Brief History of Molecular Genetic Research

The eye is the fourth most common organ to be involved in genetic disorders after the integument (skin, hair, and nail), nervous system, and musculoskeletal organs [1]. Genetic eye diseases play a major role in causing visual impairment and blindness affecting all the structures of the eye from the anterior to posterior segment. This includes diseases such as corneal dystrophy, aniridia, cataract, retinitis pigmentosa (RP), and hereditary optic neuropathy [2]. As in the case of other genetic diseases, eye diseases can also be categorized as either Mendelian, following specific pattern of inheritance, i.e., single-gene diseases or as complex diseases, where, along with the genetic components, certain epigenetic mechanisms and environmental factors also play a role in the causation of disease [3].The genetic and phenotypic heterogeneity among the inherited ocular diseases are huge, and the mutation spectrum also varies in different populations [4].

In 1943 Oswald Avery proved that deoxyribonucleic acid (DNA) carries the genetic information and later in 1953 Watson and Crick discovered the structure of DNA [5]. Twenty-two years later, in 1975 Fredrick Sanger paved the way for sequencing of DNA using chain terminator method which is popularly called Sanger sequencing. Then invention of polymerase chain reaction (PCR) technology in 1983 by Kary Mullis [6] made a revolution in the field of nucleic acid research and along with Sanger sequencing has made molecular diagnosis of many diseases facile. Thus, in the past 20–30 years, there have been considerable advances in mapping, isolation, and characterization of many genes involved in various ocular diseases such as RP, Norrie disease, choroideremia, aniridia, retinoblastoma, etc. [3, 7].

Earlier to 1990 individuals were genotyped for gene deletions or rearrangements using restriction fragment length polymorphism (RFLP) and Southern blotting techniques [8]. Then, single-stranded conformation polymorphism (SSCP) was used in identifying DNA polymorphisms. The advantage of SSCP over RFLP is that it can detect DNA polymorphisms and point mutations at various positions in a given DNA fragment [9]. This was followed by genotyping using short tandem repeat (STR) markers which allowed identification of polymorphic loci throughout the genome. The information of genome-wide STR polymorphisms used in linkage analysis allowed LOD scores to be calculated for each polymorphic locus, and a significant value of +3.00 supports linkage, while −2 or less excludes linkage [10]. With the completion of the Human Genome Project and the HapMap Project, information on single nucleotide polymorphisms (SNPs) present across the human genome were made available [11]. As SNPs are the abundant form of genetic variation in the human genome, a wide range of genetic applications have become possible [12]. In 1998 SNP array technology was developed for genotyping [13], and the first SNP array consisted of about 558 SNPs which was applied in cancer research to detect the loss of heterozygosity (LOH) and allelic imbalance in human cancers like small cell lung cancer and esophageal adenocarcinoma [14, 15]. Currently, SNP arrays have nearly one million SNPs across the genome and are widely used in gene mapping of both single-gene and complex diseases [16]. Recently, massive parallel sequencing by next-generation sequencing (NGS) technology has revolutionized the field of biology and medicine [17].

There are two approaches to identify the causative gene: phenotype-based and gene-based approaches. The first begins with the phenotype and concludes by identifying the responsible gene, whereas the other begins with the gene and works toward identifying one or more phenotypes resulting due to the allelic variation in the gene. For the phenotype-based approach, linkage analysis is carried out on large families, hunting to determine the approximate chromosomal location of the gene with the only requirement of large families with many affected individuals. In the candidate gene screening approach, the expression pattern of the gene and the protein product is characterized, and based on these functions, possible phenotypes due to the

mutations in the gene are predicted [18]. About 50 or more loci causing hereditary forms of blindness such as Leber congenital amaurosis (LCA) and RP were identified by either positional cloning or candidate gene screening approaches [19].

32.2 Homozygosity Mapping

Invention Genetic linkage analysis is the most powerful tool for the identification of disease gene. The basic principle behind this technique is that genes which are closely present in the chromosome tend to inherit together during meiosis and the traits encoded by these genes also inherit together. During meiosis, recombinants occur due to crossing over of nonhomologous sister chromatids. Closely linked genes are not separated by recombination as it happens in megabase (Mb) scale. Hence, a set of alleles tend to inherit together in the chromosomal segment, and they are usually referred as haplotype. These haplotypes are traceable in a family which is not separated by recombination. To map human traits, it is necessary to assemble family pedigrees containing a sufficient number of informative meioses, i.e., multiple affected and unaffected individuals [20]. Mapping of the genes by linkage analysis is often a difficult task due to unavailability of large families with multiple affected individuals. The invention of homozygosity mapping has been able to address this problem especially for autosomal recessive diseases. In autosomal recessive diseases, both the copies of the genes have to be mutated for the disease to manifest, and the mutation could be either homozygous or compound heterozygous (i.e., two different mutations in the two alleles of the gene) [21].

Principles of Homozygosity Mapping An increased prevalence of autosomal recessive disorders observed in genetically isolated or highly inbred families has aided in mapping genes for these different diseases. Larger segments of DNA get transmitted together in such families and exist as stretches of homozygous alleles in identical by descent (IBD) in the sibs. The extent of homozygosity, hence act as a function of linkage disequilibrium (LD) within a chromosomal region representing the recombination rate, population history, etc. Thus, it is of potential significance in gene mapping studies as they may flank rare, penetrant recessive loci. In addition to consanguineous unions, high levels of homozygosity can be expected even in non-consanguineous marriages due to the traditional practices like marriages within caste, tribes, and communities in populations like India. Therefore, comparison of homozygous blocks between the affected individuals of both consanguineous and non-consanguineous union could effectively aid in disease gene mapping for autosomal recessive disorders. Homozygosity mapping is an approach that relies on the identification of genomic blocks that have been inherited from a common ancestor (identical by descent or autozygous) [20, 22]. In a homozygous state, the regions and markers around the mutation too are homozygous, and homozygosity mapping takes the advantage of this fact to map the recessive locus or gene [21]. The runs of homozygosity spanning the candidate genes are most often compared between the (1) affected individuals of the unrelated small families (consanguineous and non-consanguineous) and (2) affected and unaffected sibs of the same family, and the genes are prioritized. Prioritization of the candidate genes in thus compared homozygous regions represents significant saving in time and resources when compared with the nontargeted sequencing of all genes in each patient [22].

History Homozygosity mapping preliminarily began with the STR markers. Initially DNA pooling strategy was employed. DNA of all affected individuals versus all unaffected individuals within the same family as controls was used [23]. With the advancement of genotyping technologies, currently SNP arrays are being used for homozygosity mapping. This strategy has been successful for mapping many

causative loci in both ocular and in non-ocular diseases [24, 25]. Alkaptonuria was the first genetic disease to be successfully mapped on chromosome 3q2 using homozygosity mapping [26] and Bardet-Biedl syndrome, the first ocular disease to be mapped to chromosome 3 in 1994 [27].

32.3 Homozygosity Mapping in Ocular Diseases

32.3.1 Retinitis Pigmentosa

Retinitis pigmentosa (RP) is a retinal degenerative disease characterized by primary degeneration of rod photoreceptors followed by cone degeneration in later years. RP presents with symptoms of night blindness, loss of peripheral visual fields, and later tunnel vision with good visual acuity in the early and mid-stages of the disease. Pigmentary changes and attenuation of the retinal blood vessels are some of the characteristic clinical features of RP. The prevalence of non-syndromic RP is approximately 1 in 4000 [28]. RP follows all the Mendelian patterns of inheritance, i.e., autosomal dominant (30–40%), autosomal recessive (50–60%), and X-linked recessive (5–15%) [29]. A few reports of digenic inheritance are also available [30, 31]. Mitochondrial mutations causing RP along with other neurological features have also been reported [32, 33], while the rest are sporadic.

RP is genetically and phenotypically heterogeneous, and till date 55 genes and three loci (without the candidate genes identified) have been identified for autosomal recessive RP (arRP) (RetNet, the Retinal Information Network). Homozygosity mapping (HM) has been extensively used along with other methodologies like candidate gene screening and recently whole exome sequencing to map the loci and identify the candidate genes in arRP [34–39]. Many studies have used homozygosity mapping as a first step of screening to know if any known arRP candidate gene is involved before searching for novel gene(s). Using this methodology, genes that were previously described in other retinal diseases or syndromic RP variants are confirmed to be causative for non-syndromic RP as well, thus adding newer genes to the repertoire [29, 38–42].

32.3.2 Leber Congenital Amaurosis

The form of congenital or early-infantile blindness known as Leber congenital amaurosis (LCA) was first defined by Theodor Leber in 1869 on the basis of typical clinical findings such as severe visual loss at birth, nystagmus, a variety of fundus changes, and minimal or absent recordable responses on the electroretinogram (ERG) before or by 1 year of age, accounting for 5% of all retinal dystrophies. LCA is usually inherited as an autosomal recessive disease, but a few cases of autosomal dominant inheritance is also reported [43]. Till date there are about 26 genes identified as causative [Retinal Information Network]. These candidate genes have been identified by using various methodologies like either by candidate gene screening or linkage studies on large families or homozygosity mapping on nuclear families using either microsatellite markers or SNP microarrays or screening genes which are involved in retinal function-/tissue-specific expression or recently by whole exome sequencing [2, 44, 45]. Out of the 26 genes, nine genes are identified either by homozygosity mapping alone or in a combination with either linkage mapping, candidate gene approach, animal model studies, or exome sequencing which has successfully identified novel LCA genes such as *ALMS*, *CNGA3*, *MYO7A,* etc. [46–52]. These genes contribute to about 50–70% of LCA, but the mutation frequency varies among different ethnic populations [53–55].

32.3.3 Bardet-Biedl Syndrome

Bardet-Biedl syndrome (BBS) (OMIM 209900) is an autosomal recessive, ciliopathic disorder characterized by RP, obesity, polydactyly,

hypogonadism, and mental retardation. Other features of varying frequency like developmental delay, diabetes mellitus, liver and heart anomalies, and behavioral problems are also observed. The prevalence of BBS varies throughout the world. In North America and Europe, the frequency is 1:140,000 and 1:160,000, respectively, postulating BBS as a rare syndrome. But in isolated/consanguineous populations, BBS is more prevalent [56]. Several approaches have been adopted to map the BBS genes inclusive of linkage analysis and comparative genomic analysis. This has been made easier with chip-based SNP genotyping where prioritization of the candidate BBS genes flanked by the homozygous tracts represents significant saving in time and resources when compared with the nontargeted sequencing of all the 21 genes in each patient [57]. Using homozygosity mapping, the candidate genes *BBS6*, *10*, *11*, *12*, *13*, *14*, *16*, and *17* have been identified. Screening and comparing 34 unrelated individuals by homozygosity mapping, disease-causing mutations were reported in 82% of the cases from India. Of these 65% mutations were novel [58]. Similar results have been reported in populations with high inbreeding/consanguinity like from Middle East, Newfoundland, Turkey, or Puerto Rico[58].

32.3.4 Corneal Diseases

Corneal dystrophies are inherited, bilateral, symmetric, slowly progressive disorders of cornea and are not related to environment or systemic factors. Corneal diseases were categorized based on International Classification of Corneal Dystrophies (IC3D) in 2008. The recent revision of the same in 2015 is based on clinical, histopathological, and genetic information and not on the anatomic site of the disease as was done earlier. They are categorized based on specific clinical definition of the dystrophy and whether the gene locus is mapped and/or the specific gene identified [59].

Congenital hereditary endothelial dystrophy (CHED) is an autosomal recessive disease of the corneal endothelium. Linkage analysis and homozygosity mapping had identified 20p13 with 8 cM interval as the locus for the autosomal recessive CHED in 1995 and 1999 [60, 61]. *SLC4A11* was identified as the candidate gene for AR CHED by candidate gene screening in 2006 and later reported in cohorts from different ethnicity [62, 63].

32.3.5 Ocular Developmental Anomalies

Perturbation in the process of eye development results in conditions such as microphthalmia, anophthalmia, coloboma, etc. (microphthalmia, small eyes; anophthalmia, absence of eyeball; coloboma, failure in optic fissure closure during development) collectively termed as "ocular developmental anomalies" (ODA) with an estimated prevalence of 1 per 7000, 1 per 30,000, and 1 per 5000 live births, respectively, in various populations [64]. The etiology of these conditions are both genetic (point mutations to chromosomal aberrations) and environmental (viral infections, teratogen or alcohol usage, vitamin A deficiency). Homozygosity mapping has been implemented in ODA, but to a limited extent [65], and the majority of mutations implicated in ODA are autosomal dominant. The technique of homozygosity mapping has been applied widely in small consanguineous pedigrees or populations with high coefficient of inbreeding. A combination of homozygosity mapping and targeted next-generation sequencing is successful in mapping many recessive mutations in small consanguineous families with ocular anomalies like microphthalmia, anophthalmia, coloboma, and others [37, 64, 66–71].

32.4 Advantages of Homozygosity Mapping

Homozygosity mapping is often the best method of gene mapping in diseases with extensive non-allelic heterogeneity. Large multi-generation families with many affected individuals are not

required as in the case of conventional linkage studies. Genotyping one or two affected individuals along with an unaffected individual is sufficient to identify the disease locus. The approach is also efficient in identifying the causative gene/mutation for a recessive disease even in outbred population in singleton cases [21]. In case of exclusion of the prioritized known candidate gene(s), the search for the novel loci/genes is easier with the available information from other homozygous regions. Many novel loci have been identified through homozygosity-/autozygosity-guided candidate gene mapping or exome sequencing [52].

32.5 Limitations of Homozygosity Mapping

Homozygosity mapping relies on the assumption that patients in a consanguineous family inherit homozygous mutation. With high allelic/locus heterogeneity, encountered for diseases like arRP, LCA, BBS, etc., this approach often remains as a challenge to detect the compound heterozygous mutations [22] or digenic variations (two different genes carrying heterozygous variation and together if present contributing to the disease) or triallelism (three deleterious allele, i.e., a homozygous mutation in a gene and a heterozygous variation in another gene). Also in small families, the chance of getting a large chromosomal segment with many candidate genes is possible which is difficult in narrowing the candidate gene [72].

32.6 Future Perspectives

Effective adoptions of homozygosity mapping in contexts characterized by low inbreeding levels are emerging in all disease conditions. One of the futuristic applications of homozygosity mapping is in complex diseases. This approach is being used in many neuropsychiatric and also in ocular diseases with familial clustering, thus suggesting the role of rare variations in these diseases.

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Tools of Genetic Eye Research and Need for Clinical Research Collaborations

33

Vanita Vanita and Umang Mathur

Abstract

Gene mapping studies offer powerful tools to identify the genes and underlying biological phenomenon for any trait governed by inheritance, including human diseases. In the 1920s onwards, human genetic variations were analyzed mainly on the basis of biochemical phenotypes by blood typing with blood group markers, blood serum protein variants, and immunological proteins such as the human leukocyte antigen (HLA) system. Beginning from the 1980s, the naturally occurring DNA variations have been used as markers to trace the inheritance in families, and it has resulted in the discovery of thousands of genes for Mendelian as well as complex diseases. With the advent of microsatellite markers, the ready accessibility of single nucleotide polymorphic markers (SNPs) through published linkage maps and public databases and automation in gene mapping technology, such as microarray systems and next-generation sequencing platforms, have made gene mapping task easier and much quicker. Also collaborations in biological science are getting bigger and more international transforming to interdependent, transdisciplinary, cross-sectional, blending basic and applied science. Along with latest approaches in gene mapping techniques, learning and applying the principles of collaborations will be an effective strategy to discover many novel genes responsible for many ocular diseases in Asian ethnic groups, whose combined numbers represent more than half the world's population.

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Eye diseases • Gene mapping tools • Congenital cataract • Retinitis pigmentosa • Microsatellite markers • RFLPs • SNPs • Linkage analysis • Whole exome sequencing • Collaborations

33.1 Introduction

Since the 1920s, studies of human genetic variations have been mainly based on biochemical phenotypes scored by blood typing using blood group markers such as the ABO, variants of blood serum proteins, and also immunological proteins, for example, the HLA system. Although Mendelian inheritance of cataract has been documented since the late 1800s, in 1963, Renwick and Lawler [1] using blood group markers (ABO, MNS, Rhesus, P, Le, L., Lu, Fy, and Secretor), described the co-segregation of dominantly inherited zonular cataract with the Duffy blood group locus (Fy), and it became the first study of mapping a human disease locus to an autosome. Also the likelihoods calculated by the authors using computerized algorithm were the first to be analyzed in this manner. Later, Donahue et al. [2] assigned the locus for the Fy at chromosome 1 that was further refined at chromosome 1q22-23 by fluorescent in situ hybridization assay [3], and a missense mutation was identified in the gene for connexin50 (*GJA8*) at 1q22-23 for this zonular cataract [4]. Similarly, different types of congenital cataracts in families of different origin were linked with the Ii blood group locus to an unknown chromosomal location that was later mapped on chromosome 6p24 [5–8]. In a Danish family with autosomal dominant congenital cataract (ADCC), the disease locus was linked to HP (haptoglobin) at chromosome 16q22 [9, 10] and autosomal dominant retinitis pigmentosa (adRP) to serum amylase locus (AMY_2) at chromosome 1 [11]. These are the initial studies of gene mapping in families with ocular diseases.

The first restriction enzyme was discovered in 1968 and utility of restriction fragment length polymorphic (RFLP) markers as genome mapping tools paved the way for the discovery of several human disease genes. Petes and Botstein [12] used DNA polymorphisms to study linkage in yeast. The identification of DNA polymorphism at the globin locus in humans [13, 14] proposed the utility of naturally occurring DNA sequence variations as generic markers to create a human genetic map and to systematically trace the transmission of chromosomal regions in families [15]. In 1983, the feasibility of genetic mapping in humans using RFLPs as genetic markers was demonstrated with the localization of Huntington disease on chromosome 4 [16]. Following this strategy, for retinitis pigmentosa (RP), the first locus for X-linked form of RP (xlRP), designated as "RP2," was localized to a DNA marker DXS7 [17] that was later refined to Xp11.3-11.23 using microsatellite markers [18]. Lubsen et al. [19] by RFLP analysis using G1 psi-crystallin gene probe reported the locus for Coppock-like cataract and of γ-crystallin gene cluster to be closely linked. An elementary genetic linkage map with nearly 400 DNA markers was generated in 1987 that expanded to ~5000 markers in 1996 [20, 21]. Physical maps providing access to linked chromosomal regions were developed in 1995 [22]. With these tools, positional cloning became possible in humans.

By lymphocyte culturing and cytogenetic techniques, Jancar [23] documented retinitis pigmentosa with mental retardation and deafness with XX/XO condition. Moross et al. [24] reported balanced translocation between chromosomes 2 and 14 [t(2;14)(p25;q24)] for ADCC of anterior polar type. Similarly, partial monosomy 21q22.2 was documented with Rieger's syndrome [25]. A reciprocal translocation between chromosomes 3 and 4 [t(3;4)(p26.2;p15)] for hereditary total cataract [26]; a de novo

pericentric inversion of chromosome 2 (p21q31) in an isolated case with nystagmus, bilateral microphthalmia, and cataract [27]; and many more chromosomal aberrations were further reported for different ocular diseases, and these studies gave clue of localization of genes for different types of eye diseases at/or near these breakpoints in analyzed cases.

Over the last few decades, the use of different types of molecular markers has played a significant role in gene mapping for different human diseases. Of these, microsatellites, term firstly coined by Litt and Luty [28], also known as simple sequence repeats (SSRs), have been utilized most extensively, as these can be readily amplified by PCR and the large amount of allelic variation at each locus. These are hypervariable, abundant, typically composed of 1–6 nucleotide repeats, well distributed throughout the human genome, and highly polymorphic. Also these are species-specific and inherited in Mendelian manner as codominant. Furthermore, the speed, reliability, and cost-effectiveness of acrylamide gel-based microsatellite analysis have made these as an attractive gene mapping tool.

McWilliam et al. [29] analyzed a large pedigree of Irish origin with type 1 autosomal dominant retinitis pigmentosa (adRP) and documented tight linkage of disease gene to D3S47, a highly informative polymorphic marker, with a LOD score of 14.7 at $\theta = 0.0$, and localized the adRP gene (RP1) to chromosome 3q. In Rieger's syndrome, for which cytogenetic investigations had indicated chromosome 4 as a candidate chromosome, by highly polymorphic short tandem repeat polymorphisms (STRP) analysis, linkage to chromosome 4q markers was identified [30]. For familial juvenile open-angle glaucoma, the first locus was mapped to 1q21-q31 by typing STRPs [31]. Armitage et al. [32] using microsatellite markers, in a genome-wide search, mapped the locus for cerulean cataract at chromosome 17q24 in a four-generation pedigree, and locus for autosomal dominant iris hypoplasia was mapped at 4q25 [33]. In a genome-wide scan, locus for autosomal dominant iridogoniodysgenesis anomaly was mapped to 6p25 [34], for autosomal dominant radial drusen to chromosome 2p16-21 [35], and gene for pigment dispersion syndrome to chromosome 7q35-q36 [36], and Young et al. [37] in a genome-wide scan using microsatellite markers mapped the first locus for autosomal dominant high myopia to 18p11.31. Similarly, genome-wide linkage analysis for diabetic retinopathy (DR) gave the evidence for linkage to chromosomes 1, 3, 9, and 12 [38–40]. Following candidate gene approach and whole-genome linkage analysis using microsatellite markers, at least 42 loci and 30 genes at these mapped loci have been mapped for isolated type congenital cataract. For various forms of retinal dystrophies, at least 278 loci and 238 genes at these mapped loci have so far been reported [41].

Although microsatellite markers are robust and highly informative, however, whole-genome genotyping is a time-consuming process. Genotyping using biallelic single nucleotide polymorphic (SNP) markers has provided an alternative strategy. De novo candidate SNPs were initially discovered by the alignment of STSs and ESTs [42–45]. A direct approach to SNP discovery was started by sequencing DNA from population-specific individuals [46, 47]. The International HapMap Project that began in 2002 sought to identify the common patterns of DNA sequence variations in human genome, their frequencies, and their correlation in the DNA samples from populations with ancestry from parts of West Africa, Asia, and Europe. SNPs are abundant and uniformly distributed throughout the human genome. The joint efforts of "The International SNP Consortium," the "Human Genome Project," and various modes of SNP discovery efforts resulted in the publication of the first genome-wide map of human genetic variations in 2007. Further, the availability of dense SNP maps coupled with recent technological developments in highly paralleled SNP genotyping has made it practical to consider the use of these markers for whole-genome linkage, whole-genome sequencing, and expression analysis using a variety of platforms. Although the heterozygosity of SNPs is lower as compared to microsatellites, however, their global genomic distribution, small amount of DNA required, improved

genetic resolution as compared to microsatellite, and adaptability to very high-throughput genotyping have made these as widely used to genome-wide analyses for various human diseases. In the beginning, SNP genotyping was reported to be carried out one SNP at a time, at a cost of ~$1 per measurement. Multiplex genotyping of hundreds of SNPs on DNA microarrays was revealed in 1998 [42], and capacity per array increased from 10,000 to 100,000 SNPs in 2002 to 500,000 to 1 million SNPs in 2007. To date the majority of large-scale genetics studies have reported genotyping 500,000–2500,000 SNPs on high-throughput genotyping arrays. Accordingly, cost per genotype has significantly reduced to $1000 per sample for a genome-wide analysis. With nanopore sequencing, industrialists and scientists are trying even more remarkable breakthrough, the ability to sequence an entire human genome for nearly $100. Genome-wide linkage analysis – based on either SNP mapping or whole-genome sequencing – is a very useful tool even in prenatal diagnostics of diseases [48].

In the past few years, using high-throughput genotyping technologies such as genome-wide association studies (GWAS), meta-analyses, and whole exome sequencing (WES), there has also been a remarkable increase in gene discovery for common eye diseases with complex inheritance patterns. The first successful GWAS was carried out for age-related macular degeneration, using nearly 100,000 SNPs tested for association in 96 cases and 50 healthy controls. Currently, by GWAS two dozen genes have been documented for myopia/refractive error. Nakanishi et al. [49] and Andrew et al. [50] for the first time by GWAS and linkage disequilibrium mapping have implicated mitochondrial and apoptotic pathways in the pathogenesis of myopia. Similarly, by GWAS over 19 genes and/loci, many of which are novel, have been associated with age-related macular degeneration. Also genes associated with primary open-angle glaucoma, angle-closure glaucoma, and glaucoma associated with exfoliation syndrome, eye color, central corneal thickness, diabetic retinopathy, myopia, retinal arteriolar microcirculation, rhegmatogenous retinal detachment, and many other quantitative ophthalmic traits have been predicted by GWAS.

33.2 Linkage Analysis for Unique Form of Congenital Cataract in a North Indian Family

33.2.1 Collaborative Research Program

Since last more than four decades, the Department of Human Genetics, Guru Nanak Dev University, Amritsar, has collaboration with Dr. Daljit Singh Eye Hospital (DSEH), Amritsar. At the DSEH, Amritsar patients from various parts of India affected with different types of eye diseases come for the clinical checkups and surgeries. We had collected a large ADCC family at DSEH under a collaborative Indo-German research project sanctioned by the Department of Biotechnology (DBT), Government of India, and BMBF, Germany.

33.3 Material and Methods

33.3.1 Family and Clinical History

The proband, an 8-year-old child (Fig. 33.1b Individual's ID: 040), was diagnosed as having congenital cataract that was phenotypically unique and was named by us as "central pouch-like" cataract, with sutural opacities (Fig. 33.1a). The detailed family history revealed 74 members in seven generations to be affected by congenital cataract. Ophthalmic examination, including slit-lamp examination and photography of lens to document the cataract type, performed on 24 family members, indicated 16 members to be affected with cataract bilaterally, and the remaining eight were diagnosed as unaffected. Cataract was of a progressive type, and cataractous changes were more prominent in affected older individuals.

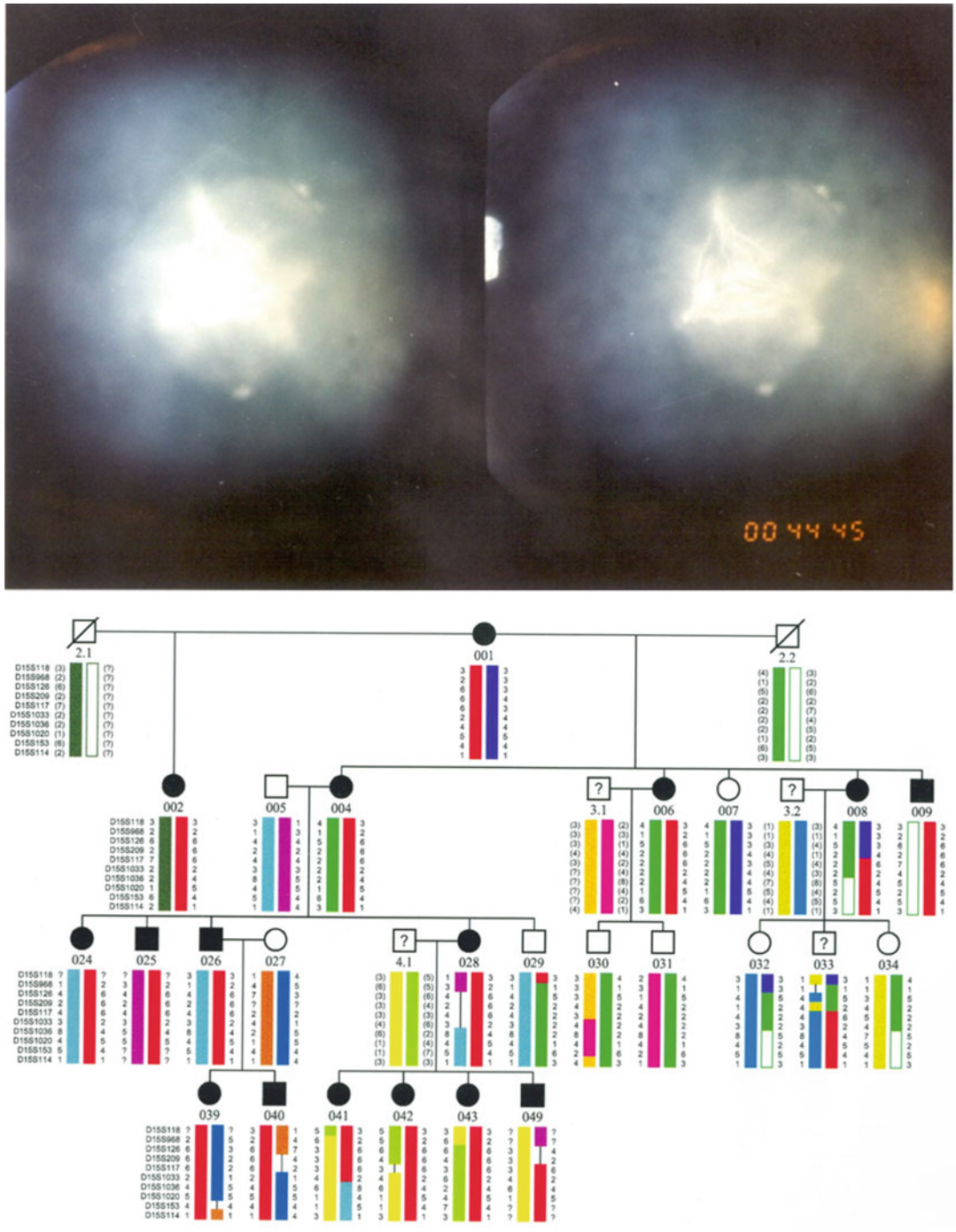

Fig. 33.1 Phenotype and haplotype analysis in congenital cataract family (CC-51) mapped to a novel locus at 15q21-22. (**a**) Slit-lamp photograph, of an 8-year-old patient showing "central pouchlike cataract with sutural opacities." Viewed in three dimensions, the cataract appeared as a six-sided central pouch, on which are seen sutural opacities both anterior and posterior, with increased prominence at their ends. (**b**) Analyzed part of pedigree of family CC-51 indicating haplotypes for analyzed markers on chromosome 15. Sequence of markers is from centromere to telomere. *Red* bars indicate the affected haplotype segregating with the disease. Inferred haplotypes are in parentheses. Uninformative markers are indicated by a vertical line in the haplotype bar. Recombination events that occurred in individuals 008, 033, 049, and 041 place the disease locus between marker D15S209 and D15S1036

33.3.2 PCR-Based Linkage Analysis Using Microsatellite Markers

On informed consent, linkage analysis using highly polymorphic microsatellite markers was performed on the DNA samples of all the 24 ophthalmologically tested individuals (including 16 affected and eight unaffected members). One individual who was recorded as affected as per family history but didn't participate in the clinical examination was also included for linkage analysis. Microsatellite markers were used for the gene mapping study and their distances were from the Généthon linkage map [21]. Markers for genome-wide scan were kindly provided by Gene Mapping Centre, Max-Delbrück Centre under our joint Indo-German project on ocular diseases. Microsatellites were amplified by Touch-down PCR (MJ-Research Watertown, MA), using fluorescently labeled primers following standard protocols. PCR products were pooled, mixed with loading dye containing internal size standards, denatured at 95 °C for 5 min, and then electrophoresis was performed on 4% denaturing polyacrylamide gels on an automatic DNA sequencer (ABI-Prism 377). The resulting data was collected and analyzed by GENESCAN, version 2.1 software and genotyping was done with the use of GENOTYPER 2.0 software. Autosomal dominant inheritance, with a disease gene frequency of .0001 and full penetrance of the trait, was considered. Recombination values (θ) were considered to be equal between males and females. Two-point and multipoint LOD score analysis was performed by means of the LINKAGE program package.

33.3.3 Mutation Analysis

For mutation screening, exon-specific primer pairs were designed for the candidate genes using PrimerSelect component of DNASTAR Program (Madison, WI). Genomic DNA from two affected and one unaffected individual was amplified. Amplification was carried out in 25 μl reactions containing 50 ng genomic DNA, 10 pmol of each forward and reverse primers, 200 nM dNTP mix, 10XPCR buffer, and 0.25 U Taq DNA polymerase (AmpliTaq Gold; ABI, Foster City, CA). PCR products were purified using Montage PCR concentrators (Millipore Corporation, Bedford, MA). Purified PCR products were sequenced bidirectionally with ABI BigDye™ Terminator Cycle Sequencing Ready Reaction Kit ver.3.1 (ABI) for a 10 μl final reaction volume, containing 5.0 μl purified PCR product, 4.0 μl BigDye Terminator ready reaction mix, and 3.2 pmol of primer. Cycling conditions were 95 °C for 2 min, 25 cycles at 95 °C for 30 sec, 52 °C for 15 sec, and 60 °C for 4 min. The sequencing reaction products were purified by the isopropanol precipitation method (ABI protocol), resuspended in 10 μl of loading buffer (5:1 ratio of deionized formamide and 25 mM EDTA with blue dextran (50 mg/ml)), and denatured at 95 °C for 5 min, and electrophoresis was performed on 4% denaturing polyacrylamide gels on DNA sequencer (ABI-Prism 377). Sequencing results were assembled and analyzed using SeqManII component of DNASTAR.

33.4 Results

33.4.1 Linkage Analysis

Initially, already reported candidate gene regions on chromosomes 1, 2, 3, 10, 12, 13, 14, 16, 17, 19, 21, and 22 were analyzed using 65 microsatellite markers, and no linkage was observed at any of these loci. Further, in a genome-wide search using 360 markers, linkage was detected with markers on chromosome 15. A maximum two-point LOD score of 5.98 ($\theta = 0$) was obtained with marker D15S117. Multipoint linkage analysis with chromosome 15 markers also supported linkage with a maximum LOD score of 5.98 at D15S117. Haplotypes were constructed for the analyzed markers on chromosome 15. One individual who as per family

history was ascertained as affected but could not be tested clinically, and hence was scored as "unknown," also shared the disease haplotype. Recombination events were detected in four affected individuals (008, 033, 041, and 049). Three affected individuals (008, 033, and 049) showed recombination proximal to marker D15S117, and the fourth affected individual (041) showed recombination event with marker distal to D15S1033 (Fig. 33.1b). No recombination was observed between markers D15S117 and D15S1033, in any of the affected individuals that indicated the disease locus at this interval. The results of both multipoint and haplotype analyses placed the cataract locus in a 10 cM interval, between markers D15S209 and D15S1036 and in proximity to marker D15S117 [51], that corresponds to the q21-22 region of chromosome 15. There has been no other previous report of mapping cataract locus in this region at chromosome 15q21-22.

33.4.2 Mutation Analysis

The genes in the proximity to the mapped locus on chromosome 15, which could be of relevance, included FBN-1 [OMIM 134797], the fibroblast growth factor FGF-7/keratinocyte growth factor (KGF [OMIM 148180]), and the gene for the orphan nuclear receptor ROR-alpha (RORA [OMIM 600825]). Therefore, to identify the disease-linked gene for this phenotypically distinct cataract, bidirectional sequence analysis was performed in the candidate genes, *FBN-1*, *FGF-7*, and *RORA* localized at the mapped interval at 15q21-22. However, no pathogenic mutation has been identified in any of these tested genes, thus excluding these as the disease-linked genes for this novel cataract phenotype. It is further planned to undertake exome sequencing on DNA samples of affected as well as unaffected members of this family so as to identify the disease-linked variation which will be further validated by Sanger sequencing technique.

33.5 Whole-Genome Mapping and Whole Exome Sequencing in an Autosomal Recessive Retinitis Pigmentosa (arRP) Family from North India

By whole-genome linkage analysis, homozygosity mapping, and whole exome sequencing, we have mapped a locus and identified the underlying mutation for autosomal recessive retinitis pigmentosa (arRP) with macular degeneration in a family from north India, who approached DSEH for ophthalmic examination. This study has also been undertaken under an Indo-German collaborative research project sanctioned by DBT, Government of India, and BMBF, Germany.

33.6 Material and Methods

33.6.1 Ophthalmic Examination

Ophthalmic examination that included visual acuity testing, intraocular pressure, fundus examination, and fundus photography carried out on 12 members of the family indicated three sibs in two sibships (Fig. 33.2a), to be affected with RP and macular degeneration. Optical coherence tomography (OCT) and electroretinography (ERG) were performed on proband(IV:1) and his unaffected father(III:3).

33.6.2 Whole-Genome Linkage Analysis and Homozygosity Mapping

A 5–10 ml blood sample was collected from each participating individual and genomic DNA extracted using standard protocol. Following quality control, HumanOmniExpress (>240,000 functional exonic markers) SNP microarrays from Illumina Inc. (San Diego, CA) were used for genotyping (IScanSQ platform) as per prescribed methods. SNP genotyping data were

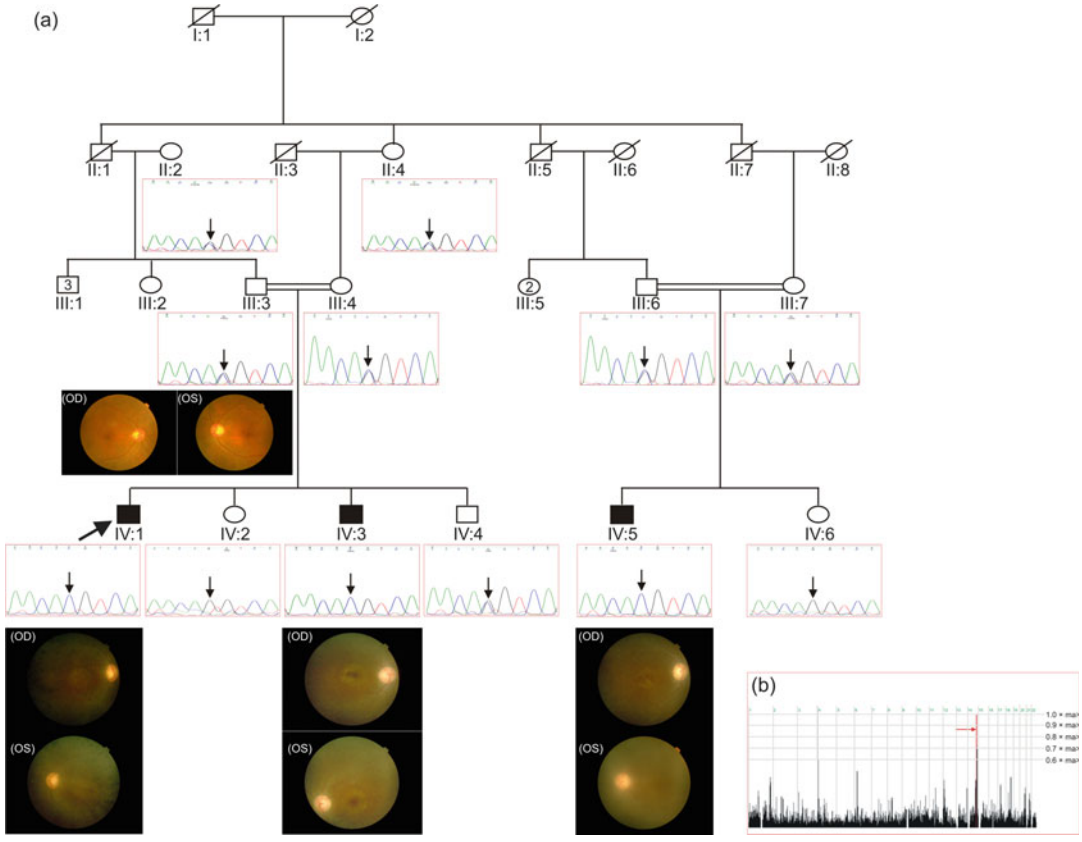

Fig. 33.2 Pedigree, fundus photographs, electropherograms, and genome-wide homozygosity mapping data for an arRP (RP-884) family with macular degeneration. (**a**) A four-generation autosomal recessive retinitis pigmentosa pedigree with three affected members in two sibships and their parents being first cousins. Individual marked with an arrow indicates index patient. Filled symbols represent affected members and unfilled symbols represent unaffected members. Fundus photograph of an unaffected individual III:3, father of the proband (IV:1) oculus dexter (OD) and oculus sinister (OS), respectively, revealed no abnormal pigmentation in the retina and no retinal changes with macula being unaffected. The arteries-vein lumen was observed to be normal (2:3); cup-disk ratio was 0.2–0.3 with a healthy neuro-retinal rim. Fundus pictures of both eyes (OD and OS) of RP patients IV:1 and IV:5 showed bilateral attenuation of the retinal blood vessels, paleness of the optic disk, bony spicules (more in IV:1 and few in IV:5) in the fundus, and macular degeneration. Fundus photograph of patient IV:3 OD and OS, respectively, depicted cup-disk ratio of 0.2 to 0.3 with bilateral attenuation of retinal blood vessels, mild pallor of the optic disk, and macula showed areas of RPE atrophy with features coinciding with macular atrophy (Bull's eye pattern) and no bone spicules (phenotype indicative of rod-cone dystrophy). Electropherogram of a part of the forward strand sequence of exon 13 of *TTC8* in all the 12 tested individuals is also presented here. *Arrow* indicated the base at which homozygous (CC) change c.1347G>C occurred that resulted in p.Gln449His substitution in all the three affected members (IV:1, IV:3, and IV:5). Electropherogram of individuals II:2, II:4, III:3, III:4, III:5, III:6, and IV:4 indicated wild-type sequence in heterozygous (GC) form, whereas individuals IV:2 and IV:6 represent wild-type sequence (GG). (**b**) Genome-wide homozygosity mapping data for the arRP-884 family with macular degeneration. The data was generated using Illumina 8-sample Human OmniExomeBeadchip. HomozygosityMapper plot indicated the homozygous region at chromosome 14, shared by all the three affected individuals (IV:1, IV:3, and IV:5) (*red line*, indicated by an *arrow*)

analyzed using HomozygosityMapper [52], and haplotypes were constructed with ALLEGRO [53] and presented graphically with HaploPainter [54].

33.6.3 Whole Exome Sequencing

WES was performed on a DNA sample of the proband(IV:1). The DNA sample was captured using SureSelect Human All Exon KitV2 (Agilent, Santa Clara, CA, USA), and sequencing of 100 bp paired-end reads was carried out on a HiSeq 1500 (Illumina) platform. Sequence reads were mapped to the haploid human reference genome (hg19) using BWA [55]. Single nucleotide variants and short insertions and deletions were called using GATK [56]. Variant annotation on functional level was carried out using Jannovar [57]. Variant and gene prioritization were performed using the Exomiser [58] and ExomeWalker [59]. A total of 51 genes previously known to be linked with different forms of RP including *DHDDS*, *RPE65*, *ABCA4*, *PRPF3*, *SEMA4A*, *CRB1*, *USH2A*, *ZNF513*, *C2orf71*, *FAM161A*, *SNRNP200*, *MERTK*, *CERKL*, *SAG*, *ARL6*, *IMPG2*, *RHO*, *CLRN1*, *PDE6B*, *PROM1*, *CNGA1*, *PDE6A*, *MAK*, *TULP1*, *GUCA1B*, *PRPH2*, *EYS*, *KLHL7*, *RP9*, *IMPDH1*, *RP1*, *C8orf37*, *TOPORS*, *CDHR1*, *RGR*, *BEST1*, *NRL*, *RDH12*, *TTC8*, *NR2E3*, *CNGB1*, *PRPF8*, *CA4*, *PRCD*, *FSCN2*, *PDE6G*, *PRPF31*, *IDH3B*, *PRPF6*, *RPGR*, and *SOX3* were used as seed genes.

33.6.4 Mutation Analysis

Mutation detection in the candidate gene was performed by bidirectional sequence analysis. Initially, genomic DNA from proband and an unaffected individual was amplified in 15 μl reaction using standard protocols. Amplified products were purified and sequenced bidirectionally using BigDye Terminator Cycle Sequencing Ready Reaction Kit ver. 3.1 (ABI) following standard protocols, and electrophoresis was performed on 3500xL Genetic Analyzer (ABI, Foster City, CA). Sequences were assembled with SeqA6 software and analyzed using SeqScapev3.0 software.

33.7 Results

33.7.1 Ophthalmic Findings

Fundus examination conducted on 12 available family members revealed attenuated retinal blood vessels, “bone spicule” pigmentation in the midperiphery of the retina, macular degeneration, retinal pigment epithelium (RPE) degeneration, and waxy pallor of the optic disk, in three family members (Fig. 33.2a), and hence scoring them as affected with RP and macular degeneration. Age of onset of RP and loss of central vision in the patient IV-1 was at 17 years and 18 years of age, in patient IV-3 it was at 16 years, and at 2 and 5 years, respectively, in patient IV-5. Visual acuity was in the range of <6/60 (OD) to 6/36 (OS) for affected individual IV-3 and 6/60 (OD) to <6/60 (OS) for affected individuals IV-1 and IV-5. High myopia was also observed in all the three patients. The father and mother of the patients did not show any retinal changes on fundus examination. OCT of the one patient showed fraying of the rod and cone layers, hyperflexed areas in the RPE layer (due to accumulation of pigments), and macular degeneration as compared with his unaffected father. ERG testing of proband showed extinguished rod and cone response, whereas his unaffected father showed increased implicit time and reduced amplitude in scotopic 0.01 ERG indicating carrier state. None of the three affected members showed any extraocular characteristics and hence were considered to be affected with non-syndromic arRP with macular degeneration.

33.7.2 Whole-Genome Linkage Analysis and Homozygosity Mapping

Genome-wide genotyping data that was generated by SNP microarray analysis of three affected and four unaffected members of this

arRP family revealed a single homozygous region at chromosome 14 (Fig. 33.2b: red line and red arrow). Haplotypes generated for the analyzed SNPs at the mapped interval on chromosome 14 revealed a recombination event that would have occurred in two affected individuals at SNP markers rs7156613 and rs7159552, respectively, and placed the critical interval in a 4.47 Mb region between rs7156613 and rs7159552. All the tested SNPs within the interval rs7493702 to rs8003022 were homozygous in all the three tested affected individuals.

33.7.3 Whole Exome Sequencing

WES carried out on proband indicated a total of 215,700 variants, 34,222 of which were on the exome target. Initial analysis was carried out using the Exomiser to restrict candidate genes to the linkage interval between SNP markers rs7156613 and rs7159552 (chr14:85566856–90106316; genome build GRCh37) and to filter out variants with a population frequency of 0.5% or higher. This left *TTC8* as the only candidate gene with a homozygous variant at chr14: g.89338796G>C. This substitution seems to lead to a missense change at position 449 of the TTC8 protein, corresponding to NM_144596.3: c.1347G>C;p.Gln449His. In addition, exome-wide prioritization carried out using ExomeWalker taking 51 genes associated with RP as seed genes, again, flagged *TTC8* as the most likely candidate gene.

33.7.4 Mutation Analysis

Mutation screening in exon 13 of *TTC8* revealed a previously unreported substitution, i.e., c.1347G>C (p.Gln449His) [60], in homozygous (CC) form in all the three tested affected individuals of this family, whereas nine unaffected individuals were either wild-type homozygous (GG) or heterozygous (GC) (Fig. 33.2a). The c.1347G>C substitution was not observed in 100 ethnically matched controls (200 chromosomes), hence excluding it as a polymorphism. The mutation affected a highly conserved position of TTC8. c.1347G>C affected the last nucleotide of exon 13, thereby affecting the 5′ splice site consensus sequence MAG|GTRAGT (where M is A or C and R is A or G). Analysis using the splice site prediction by neural network application of the Berkeley Drosophila Genome Project [61] indicated a reduction of the donor site splice score by 0.90 to 0.42. There are previous reports of apparent missense mutations affecting the last nucleotide of an exon, however, actually leading to missplicing [62]. RNA from RP patients was not available to test the effects of c.1347G>C substitution on splicing; however, it seems possible that the variant exerted pathogenic effects either by the amino acid substitution or by an effect on splicing or both.

33.8 The Need for Collaborations

In the recent past, collaboration in biology, specifically in genetics, has increased substantially. Much has changed from the time Watson and Crick gave the 3D structure of DNA in 1953. Watson's "double helix" recounts that a limited number of scientists worked relatively independently within a small-scale academic environment of the Cavendish Laboratory in the traditional English university town Cambridge [63]. In contrast, John Sulston in decoding the human genome illustrates a different world of hundreds of scientists across the globe working together on a dynamic project [64]. Scientific collaborations have increased as is evidenced by an increase in average team size [65, 66]. Collaborations are getting bigger and more international. Single investigator studies within a laboratory are transforming to interdependent, transdisciplinary, cross-sectional, blending basic, and applied science.

33.9 Why Collaborations Are Necessary

Geographically scattered distribution of research material for genetic studies is the prime reason

for research collaborations. Also, understanding the role of environment on expression of gene has become an important issue in comparative genomics. It requires populations with genetic and environmental diversity that cannot be satisfactorily attempted within the confines of one region or one country alone. The current knowledge of ethnic variability in the epidemiology of major eye diseases in Asia is limited. The Asian Eye Disease Consortium aims to evaluate the prevalence, risk factors, and impact of major eye diseases in Asian ethnic groups, whose combined numbers represent more than half the world's population.

There is a need to connect basic research to translational medicine through collaborations with clinician scientists and PhDs. Basic science is extremely important for advancing general knowledge; however, there is a need to apply it to solve real-world issues like those of human disease and global health. It is a relatively unmet need in the academic setting.

Collaborations in biological science are driven by the need to accelerate research to improve human health and well-being. Societal factors, together with an increasing political emphasis on the relevance and application of research, have combined to increase markedly the magnitude and complexity of collaboration in biology. Additionally, funding incentives can stimulate collaborations. Funding agencies across the world, such as government and private foundations, are increasingly structuring request for proposals to favor the involvement of interdisciplinary research teams.

Collaborations in biology have a more networked structure. The dispersed nature of genetic material may necessitate collaborations with physicians in remote locations, while scientists in their laboratories have capability to process a large number of samples. Decreasing travel and communication costs gives an opportunity for scientists and clinicians to work together. Standardization of data and findings enables discovery of new patterns and generating new hypothesis to improve our understanding of disease.

One important justification for collaboration is the enhanced ability to share and exchange resources. Shamoo and Resnik [67] have defined "data, databases, ideas, equipment, computers, methods, reagents, cell lines, research sites, personnel, and many other technical and human resources" as resources. Benefits from collaboration may include cost savings and the potential to facilitate scientific progress. Collaborations between different streams of science and medicine are opportunities to learn from different disciplines. The intersections of disciplines are places where new ideas emerge and innovative research happens. Analytical frameworks and the tools of one discipline are often relevant to other disciplines and can revolutionize the research that can be done.

Collaborative papers tend to get cited more often. Changes in the balance of research done by the lone scientist and that done by teams can be seen in coauthorship data [68]. Coauthorship has been increasing inexorably and recently it has exploded [69]. This upward trend in multiauthorship in biological sciences will continue through shared global priorities in health. Collaboration can also be useful in establishing innovative alliances between research teams from academic, government, and private industry. These alliances can result in long-term research relationships benefiting science and society, with broad-based economic interests. Both science and society are best served by collegiality and open collaboration [70].

33.10 Creating an Environment for Collaborations

Niessen and Krieg, [71] in their editorial in the "Journal of Investigative Dermatology," mention that the differences in training background, research approach, and career perspective – and sometimes a lack of true mutual respect – often stand in the way of more productive MD and PhD collaborations that benefit both science and clinic. Many basic scientists working to unravel the molecular mechanisms of human diseases

never have the opportunity to interact with people who suffer from or treat those diseases. PhDs receive little training in translational research and are poorly equipped to identify and ask clinically relevant problems and questions because they do not necessarily incorporate patient characteristics and clinical observations into their thinking. Likewise, physicians have in general received little formal training in the rigor of scientific questioning and thinking, and during their training, they have not been exposed to the same range of techniques as PhDs, necessary to address basic scientific questions.

We must acknowledge and reward the different but essential contributions made by both PhDs and clinician scientists to science and recognize that it will be crucial to promote collaborations. The Human Genome Project is often viewed as the first true large-scale collaboration in biology, giving rise to a variety of publications discussing issues related to collaboration such as structure, data exchange, and public-private competition [72–74].

Good scientific collaborations require strong interpersonal relationships and deep emotional commitments to the group. In pursuit of a common goal, researchers engaged in collegiality treat each other with respect providing constructive criticism as well as assistance. Strong leadership is a prerequisite for promotion of collaboration in research. Leaders need to have a clear vision and direction for science, analysis-based priorities and decision-making criteria, and sustained follow through. They need to encourage a culture of creativity and innovation with a willingness to take risks while maintaining the values of integrity and excellence. They need to safeguard against a culture of competition across and within units and its replacement with a commitment to the greater impact that is possible with an integrated approach.

While science can benefit from the practice of collaboration, there should be good agreement about the appropriateness of methodologies and analyses in the team, to avoid acrimony. Clear and relevant study questions help in keeping focus as well as avoid confusion among the researchers.

Technology can help in improving communication and sharing of information, even in remote locations. However, relying too heavily on technology to promote communication is no substitute for a shared commitment to accountability in following through on all assigned tasks. Fostering partnerships requires mechanisms to facilitate linkages internally and externally. Organizing communication is critical to success by facilitating the identification of opportunities for collaboration and sharing information, perspectives, ideas, technologies, and new knowledge.

Research projects need to invest in full-time, qualified, professional managers at the central and unit levels. The management needs to provide enabling administrative systems that support and facilitate interdisciplinary scientific research (and not create hindrances).

33.11 Ingredients of a Successful Collaboration

To make collaborations work and contribute scientifically, understanding of collaboration's dynamics is essential. Preparing ground rules early in collaboration; assigning everyone's role and duties; issues like data collection, data analysis, procedures to verify every collaborator's data, sharing of data, communicating results, authorship, participation in joint activities, meetings, reliability, and realistic approach; and administrative management are crucial for successful collaboration. Mechanisms should be put in place to exclude opportunities for research misconduct. It may include involving someone independent to verify data without anyone feeling offended. As with any relationship, collaboration means sharing the rewards but also sharing problems and issues.

It is critically important that all scientists assume responsibility for the integrity of their published work and ensure that they are properly acknowledged for their contributions. The editorial in Nature Chemical Biology states that most authorship disputes result from lack of clarity on two main points: firstly, defining whether an

individual's scientific contributions warrant authorship and, secondly, determining the order of the author list [75]. Collaborative research teams should explicitly discuss authorship at the outset of multigroup projects. Clear expectations and open dialog will not prevent all authorship misunderstandings, but they should provide channels necessary for resolving them.

The desire for financial gain can potentially conflict with biomedical researchers to conduct studies according to scientific and ethical principles. Intellectual property rights play a central role in today's biomedical research. Discussion among the collaborating institutions on source of funding, distribution of funds, and financial conflicts of interest as well as on intellectual property is important. Creating a transparent environment for collaboration with high ethical standards may lead to trust and long-lasting relationships.

33.12 Challenges in Collaboration

Research is undoubtedly a competitive activity and much "work in progress" is conducted in secrecy until it reaches a stage where priority can be claimed. Competition should give way to collaboration, on agreed terms, among the group. It is important to recognize that together they would reach the common goal faster than by working in isolation.

Ethico-legal frameworks, responsibilities, and obligations of using human research specimens transported across collaborating institutions can be challenging. Collaborations have grown in size and complexity with the advance of molecular, genomic, and post-genomic research. The challenge is to satisfy legitimate public concern about ethics of use of human sample and protecting human dignity and not inhibiting innovation and discouraging investment in research. In case of international transfer of biological material, care should be taken that knowledge/expertise of local nationals is not exploited to access local biological materials. Procedures to ensure safe transfer of samples from one institution to another (particularly with regard to preservation of the tissue donors' confidentiality) would also need to be ensured.

33.13 Summary

For the past decades, gene mapping techniques such as linkage analysis and candidate gene approach have been successful in elucidating the genetic defects underlying various ocular diseases having Mendelian pattern of inheritance. In contrast, complex ocular diseases have been more difficult to unravel using linkage approaches. On completion of the human genome project and the cataloguing of millions of common single nucleotide polymorphisms, and with the advent of large-scale genome-wide association studies, powerful meta-analyses, and next-generation sequencing technologies (which can test the genome at the resolution of a single base pair), have led to unparalleled advances in revealing the genetic contributions underlying complex disease as well. However, genetic mapping is only a first step toward biological understanding of any disease. Investigation of functional roles of identified genetic variants is vital in exploring disease pathogenesis so as to pave the way for translation of genetic data into clinical practice for the diagnosis and development of gene-based screening tests, targeted gene therapies, and preventive strategies. Therefore, there is a strong need to connect basic research to translational medicine through extensive collaborations among clinicians and researchers.

Compliance with Ethical Requirements Vanita Vanita and Umang Mathur 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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Eye Genetics: The Road Ahead to Quality Standards

34

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Abstract

The prevailing system of running research labs in conventional set-ups are based on no uniform method of management, relying completely on individual preferences and experiences. Unfortunately, even the leading institutes involved with research in health and medicine fail to incorporate defined modules unless mandated by the guidelines of GMP, essential for clinical translation. The present trend shows the drop in clinical trials presumably due to inherent questionable data and lack of back traceability of information. The ability to bring clinical level safety at the genetic research level facilitates Quality control measures unique, transparent and credible for clinical translation. Good Laboratory Practice (GLP) modules have been developed to enhance quality standards, increase credibility, efficiency and transparency of research settings. The studies in ocular genetics, pathology, pharmacology require similar benchmarks. There have been rapid increase in the cases of eye diseases in Asia but the process of genetic data generation is not uniform and auditable. The studies involving larger populations include meta-analysis and GWAS etc. which require collaborations, need to be supported by a standardized managerial system. Quality standards define such tools ensuring back traceability, verifiability and auditability of data and test systems. These should also address IP conflict management and implementation of standardized protocols. The quality control

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and GLP can lead to benchmarking of research data which are usually considered a soft activity in Medical Institutes where most of the genetic data is generated. The Quality control practices in such large scale research collaborations require testing the source, prevalence and resulting therapies for overall ocular health.

Keywords
Quality assurance • Good laboratory practices • Eye genetics

Around 285 million people over the world live with visual deficiency. Out of these 39 million people are blind, and 117 million are with uncorrected refractive errors [1]. Most of the blindness is curable. India is now home to the world's largest number of blind people with more than 18 million people who suffer from one or other form of blindness. Genetic factors are important in the development of eye diseases. An increasing number of genes have been associated with eye disorders. The delivery of accurate test results is the most serious problem indicator for laboratories. Presently, practice of medicine has been entirely redefined by the developments in genetic testing. Genetic testing is widely used as common tools by research institutions. However, there is extensive and widespread application of genetic testing and analysis after the completion of the Human Genome project. During genetic testing, there is always a possibility that any systematic or random error may occur and put human health at risk. To categorize and to work with eye genetics, we need to build and improve the quality standards with genetic research and services to achieve excellence.

34.1 Quality Throughout the Testing Process

Genome-wide association (GWA) and meta-analysis have accelerated the pace of genetic research in eye diseases. GWA studies are based on analysis of single nucleotide variation in the genome and its association with pathological condition. GWA studies are required to have case and control participants. Case group may be clinically pre-diagnosed, but control group should not have similar disease phenotypes. After the completion of human genome project (HGP) in April 2003, it identified millions of SNP changes in the whole genome which has been deposited in public database "The SNP consortium (TSC)." Two major aspects need to be emphasized in GWA studies, i.e. large population size and large genome coverage. Large population size is important to avoid false-positive genetic association derived by modest p-values for some of the genetic alleles. Similarly, a genotyping array provided by companies (like Illumina, Perlegen, Affymetrix, etc.) does not cover the whole genome. Meta-analysis is quantitative statistical analysis from all the studies done in particular field by combining them all and to draw a precise and effective scientific inference. Meta-analysis shows the presence of heterogeneity by including all relevant studies randomly that could provide impartial and balanced conclusion. Even the studies which have less impact due to less sample size could show up on meta-analysis inference. Meta-analysis studies provide new research question or perspective which assist policy makers to customize new policies/protocols in the research area. Therefore, in both kinds of studies, it is crucially important to have proper guidelines or checklists to improve the quality of such studies, reports, and scientific conclusion obtained from these reports. Here in this chapter, we have discussed some of these issues which should be included while performing such studies.

34.2 GWA Studies in Eye Research and Its Quality Control

GWA studies could provide direct relation between disease and genetic changes, which possess powered study designs. There are some limitations in GWA studies, which result in drawing varying inferences. However, we can enhance the quality of GWA studies by considering following issues in various studies.

1. GWA studies have defined case-control population in which case could be pre-diagnosed before recruiting them in the study, while control population must not have similar disease phenotypes as case population. There should be proper documentation of inclusion and exclusion criteria in verifiable format.
2. Sample size for GWA studies is important since all its inference and conclusion are based on the significant association of SNP variance with disease pathology (p-value). Moreover, there are a few studies which have been retracted from renowned journals due to discrepancies raised by use of different kinds of genotyping arrays. Commercially available high-throughput genotyping array (e.g., *Affymetrix*, *Illumina*, or *Perlegen*, etc.) covers only a small proportion of total SNPs found in human genome. Various arrays used in genotypic analysis have different sets of SNPs, which lead to different inference/conclusions and also have reported various genetic markers in disease pathology. In the Indian set-up, such limitations can be addressed by adopting whole genome sequencing or next-gen seq. method. Additionally, the introduction of statistical imputation analysis in such studies could bridge the gaps for missing genetic links between different study outcomes. The cost of sequencing is now much cheaper and can be made affordable in Asia if quality tools are implemented.
3. The whole genome coverage analysis also provides gene-gene interaction and assortment of different genetic alleles in disease phenotypes.
4. GWA studies also are not able to describe the cross talk between different diseases and their common SNPs. The pleiotropic effect of particular SNP which affects different sets of other disease conditions cannot be analyzed by GWA studies.
5. GWA studies must also have to include some statistical tools that could analyze the gene-environment interaction and epistasis phenomenon in disease phenotypes. Such problems are being solved by introducing genetic linkage analysis and Mendelian randomization approach.

34.3 Quality Control in GWAS

34.3.1 Sample Quality

It is very crucial to code the sample immediately on receipt along with defined gender identifier. The chromosomal structural changes and atypical X chromosome numbers can also influence the end point analysis of GWA studies calculated through software. It is very important to code the sample properly and also introduce basic questionnaire, which includes all the basic points like sex and other sociodemographic details of the participants. By doing so, it could reduce the chance of type 1 and 2 statistical errors in final analysis of the genetic data. However, sample identity and kin relation could also be identified by using PLINK software while analyzing GWAS genetic data. Pairwise dot matching in the dot plot of GWAS data could identify unrelated, parental, or duplicate/twin samples by their scoring as 0, 1, or 2, respectively. The graphical representation of the pairwise data can be done through R software [2, 3]. It is also possible that in such studies, an individual can be involved in more than one center in case GWA studies are multicentric. The common method used to identify the ancestor in a large population is principle component analysis (PCA). Therefore, in such cases, the person has to be excluded after software analysis by which we can reduce the type 1 and 2 statistical errors in final analysis.

Additionally, it is also desirable to assign a defined sex or number of X chromosome of the participant so that it may not increase the copy number variance or the SNP's number of total genome as in case of Klinefelter's syndrome (XXY) or Turner syndrome (XO). The X or Y chromosome numbers can be calculated through probe intensity or log-R ratio which is the ratio of a particular sample to total samples. If the value is less than zero, it is considered as deletion in the chromosome, and a higher than zero value could be signified as the duplication of chromosomal genes or nucleotide sequences throughout the chromosome. Considering these issues, the quality of analysis can be enhanced. Therefore, a good way is to maintain a checklist in a lab and ensure periodic orientation of research staff associated with genetic analysis.

34.3.2 Population Stratification

Population stratification is the major problem of biasness in the population-based case-control study, which has different genetic and phenotype appearance [4]. Therefore, it is advisable that the population must be homogenous in nature in GWA studies. True-false association occurs due to the ancestry rather than the true association of allele to disease. Small stratification in population affects GWAS severely because large population size is required to detect common variant of complex disorders [5]. By combining the samples from multicentric GWA study, the complete analysis could result in population stratification. Population stratification could be detected and adjusted by applying genomic control approach [6, 7]. Tools like Eigenstrat analysis [8] and STRUCTURE [9, 10] could be used to address the biasness raised because of population stratification by combining data from different subpopulations and further linked with the data of cohort GWA studies.

34.4 Meta-analysis and the Contribution in Quality Control

The meta-analysis studies provide scientific clues in a particular field by combining all the relevant articles. It provides the precise and effectiveness of the study which had even shown less significance in an individual study. Hence, such studies are very useful in policy making and initiation of evidence-based treatment [11, 12]. As compared to GWA studies, the heterogeneity in literature selection can play a crucial role in conclusion of the study. Therefore, quality control in meta-analysis is an important component in order to provide impartial, balanced, and effective conclusion of the research done in the particular field.

There are some issues that may prevent biasness from the meta-analysis.

34.5 Random Selection and Independent Literature Search

A good and independent literature search is important to reporting the accurate conclusion in meta-analysis [13]. Literature search should not be confined up to only single database. Literature search and selection of article could be done on different search engines independently like PubMed or MEDLINE or Embase with three independent key words by each individual author.

34.6 Inclusion and Independent Review of Selected Articles

It is also important to isolate relevant article in the study and that should be reviewed by each authors to make their critical comments on inclusion and exclusion of the paper.

34.7 Data Extraction

Most data extraction analysis is based on the question to be addressed by the meta-analysis. Therefore, it is necessary in such studies to extract data independently with multiple individuals. It is useful to compare the results obtained from independent data extraction and resolve any divergence in the results or conclusion.

34.8 Analysis and Conclusion of Meta-analysis Studies

More rigorous and complex analysis is required in such studies, especially when it includes a large number of studies to avoid biasness in the conclusions of study i.e. there must be heterogeneity in data analysis. Heterogeneity is defined by how many variables are analyzed in the studies in order to make a harmonious conclusion. Heterogeneity could happen at the level of mythology, too. Moreover, the type of statistical analysis could also create statistical heterogeneity. Therefore, in any meta-analysis, it is imperative to report what kinds of approaches have been used in interpretation and analysis of data.

There are several guidelines statements being used. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [14] statement defines the guidelines to carry out the meta-analysis especially in clinical trials. Similarly, Meta-analysis Of Observational Studies in Epidemiology (MOOSE) [15] statement has also described the guidelines for epidemiological studies and their conclusions. These essential guidelines and protocols have also been described in "Cochrane Handbook for Systematic Reviews of Interventions" [16]. Recently, the Quality Of Reporting Of Meta-analyses (QUOROM) and checklist of meta-analysis statement provide descriptive protocol to present abstract, introduction, methods, results, discussion, and conclusion of the meta-analysis studies. It also defines the method of searching, inclusion, and selection of articles. It contains the checklist for inference, data assessment, validation, data abstraction, and extraction. By failing to comply to the QUOROM checklist, it could enhance the biasness of meta-analysis study.

34.9 Confidentiality and Security of Eye Genetic Research Data: A Case for Global Quality Control

Emergence of techniques to explore the human genomic information in order to provide the health care services and for the interoperability of information has increased the vulnerability to privacy breach. Patients' genetic data has direct implications on the family members at risk due to which exchange of genetic information needs to be regulated by timely documentation and installation of tools for protection of privacy, ensuring regulated health-care delivery. There is a need to have global law that ensures compliance by signatory nations so that confidentiality of patients is protected.

34.10 Definition of Genetic Research Data

Nucleic acid information includes targeted diagnostics, where a single gene or a polymorphism is evaluated; population-based newborn screening by MLPA, NGS, or PCR; and large scale microarray techniques which screen multiple genes and polymorphisms constituting genetic data. Analyzing human DNA, RNA, and banding patterns that aid in providing diagnostic and prognostic information about an individual or a patient needs regulation because more and more eye-related disorders are being discovered that have genetic basis [17].

34.11 Consent for Sharing

Most informed consent forms for data collection state that the information obtained will remain confidential, and it is made sure that disclosure is done with precaution. Though genetic information

constitutes medical information for individuals and families, there is a conflict between maintaining confidentiality with the family [18].

34.12 Storage

Most of the genetic information is preserved in the form of electronic data, which must be kept in server-based storage, and data accessibility should be strictly through virtual private network (VPN) in the secured network. Server domains are required to be allocated to the staff so that personal confidential data can be stored with restricted accessibility and back traceability.

34.13 Good Lab. Practices

Implementation of GLP principles has the potential to develop a system-dependent management besides delivery of diagnostic services and storage of genetic data in the secured places. Some of the GLP practices lacking in India must be followed as below:

1. **Master Schedule:** Each research staff could prepare a weekly plan of genetic analysis work to be completed in a month. Master schedule can then be submitted to quality assurance (QA) before the beginning of the month. QA can then review the progress of the research and technical staff at the end of the month.
2. **Quality Assurance**: Independent study, infrastructure and facility-based QA reviews could be carried out once in a month to record and monitor the deviations in genetic analysis. Study-based QA inspections can monitor the work as per their master schedule. Deviations from the master schedule can be documented and followed up in the next month. Independent checks of experiment are carried out to check the compliance to SOP.
3. **Formulation of Documents:** Standard operating procedures (SOPs), data recording sheet (DRS), and raw books can be maintained by QA and provided to research worker from time to time. Any experimental procedure performed in the laboratory could thoroughly adhere to respective SOPs enabling verifiability and back traceability of data and procedures carried out in lab from time to time. Any deviation can then be reported in DRS as well as raw book which could be the source of information for identification of problems. SOP and DRS should be stored in secured premises. Such systems can automatically impart quality checks at various procedures.

Standard Operating Procedures (SOPs) SOP can be prepared to streamline the experimental protocol and make administrative functioning of the genetic unit transparent and auditable. This may include specifications of experiments, along with time-dependent log sheets of chemicals and equipments used in the procedure. It facilitates the system dependence of research workers and reduces the scope of errors or confidentiality breach in handling genetic data of eye disorders. Any experimental and administrative procedure performed in the laboratory should adhere to respective SOPs approved by the management.

Data Recording Sheet (DRS) To minimize the procedural errors, DRS could be formulated for the real-time monitoring of genetic analysis. DRS can be prepared in consultation with study director and typically consist of procedures for identification of SOP, patient coding, sample locations, reagents used, and document control with assistance of QA. DRS can be issued only at the time of conducting the experiment and resubmitted to the QA after approval from the study director.

Personal Discussion and Lab Meeting A day in a week/month can be allocated to every research worker for quality discussion regarding the genetic testing platform, research problems, progress, and validation of vendors from which purchases are being planned so that there is continuous communication and sharing of information to avoid confusion and mismanagement.

Logbook Logbooks for each instrument used in genetic analysis could be helpful in not only maintaining the record of usage of resources but also ensuring error reporting and back traceability of data.

Log Sheet Log sheets can be helpful in organizing resources and their utilization in a timely manner. This may contain vendor information, expiry date, total amount, purchase information, and stock entry information.

34.14 Formulation of Checklists, Price list/Benchmark/ Instrument Files

Checklist Checklists can be developed to ensure the completeness of the task assigned to research workers, which compensate for the human limitations. Checklists can be formulated for all of the academic or administrative lab procedures including consent forms, human ethical clearance, and storage of research data as well as confidentiality proformas.

34.15 Instrument File

A logbook can be installed along with the instruction sheet and equipment maintenance file applicable to the experimental protocol at hand. Any experimental procedure performed by the use of these instruments in the laboratory can be adhered to, by following the respective instruction sheets. Any deviation can be reported in the logbook as well as instrument maintenance file which could be the source of information for identification of problems in the instrument. Instrument file can include calibration chart; service and maintenance chart; installation, operational, and performance qualifications; instrument manual; instrument password; and information of person to be contacted in case of emergency.

4. **Auditing:** Internal and external auditing of genetic data enhances the accountability and acceptance of the study. The quality assurance (QA) could conduct periodical audit of the progress and compliance and reproducibility of experimental protocol. The data generated can be filed in a defined format using raw book, calibrated instruments (with IQ, OQ, PQ), master code, log sheets, and regulated room environment providing backup for each facility (including power outrage). Validation of data from an independent lab is desirable in order to enhance transparency.
5. **Archive:** The archiving facility coupled with quality assurance program can allow maintainability of important records and samples, thus reducing the time of both the research personnel and patients.
6. **Master Coding:** Coding chart can be developed by laboratories to mask the direct sample information in the format, which can be easily decoded. Once a human sample enters in the laboratory, it must be made mandatory to code the samples in order to protect the confidentiality of valuable genetic information.
7. **Periodic Meetings:** Research workers who deal with genetic information can conduct periodic meetings to discuss the problems of genetic analysis in the experiments, storage, ethical issues, and compliance to quality control-related issues which can then be recorded and documented in the defined formats to further link the information in the common folders.

34.16 Conclusion

Quality control covers periodic training, knowledge of bioethics, confidentiality, and ownership of inventions, which is essential for supervising data quality. With the advancement of high-throughput genotyping and sequencing techniques together with improved analytical methods, the contributions of genetic and

environmental factors in the development of eye diseases needs to be clarified. Yet much remains to be explored and more quality control with appropriate applications is needed in genomics so that the data generated is helpful in practicing precision medicine. The ultimate goal is the development of a panel of quality standards for genetic testing in eye disorders in order to improve patient care and research.

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Genetic Counseling in Asia

35

Viney Gupta and Ken Nischal

Abstract

Genetic counseling in Asia still lags behind that available in developed countries. Not much emphasis has been given on genetic education in most Asian countries, although they have the greatest burden of genetic eye disorders. We review the magnitude of the problem of genetic eye disorders in Asian countries and the current scenario of genetic counseling available.

Keywords

Genetic counselling • Eye disease • Genetic education

35.1 Magnitude of Ocular Genetic Disorders in Asia

Genetic disease is the most common cause of blindness in infants and children in developed countries, accounting for approximately 50% of all childhood onset blindness [1]. It also represents a public health and social and economic problem for developing countries. The global prevalence of blindness in all ages was estimated as 0.58%, with the highest percentage being reported for China (20.9%), India (20.5%), and African region (12.5%) [2]. Asia homes for half of the world's 40 million blind population. In many developing countries of Asia, congenital eye disorders have high prevalence mainly due to high rates of consanguineous marriages, high birthrate, and a lack of expertise in genetic counseling [3, 4]. Our understanding of the genes involved in inherited eye disease (IED) is now sufficiently advanced, and molecular technology is sufficiently powerful that we can hopefully expect the deployment of useful tests for nearly all IED during the next 5–10 years.

The pattern of eye diseases varies in racial/ethnic groups and exhibits a different pattern in Asian countries. However, the current knowledge on ethnic variability in the epidemiology of

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major eye diseases in Asia is limited. Even within Asia there is a huge variation. High prevalence of myopia, higher incidence of acute angle-closure glaucoma, and higher rates of retinal detachment are observed in Chinese rather than Indian and Malay population [5–8]. The prevalence rate of early and late age-related macular degeneration (AMD) was reported to be 10.3% and 1.1%, respectively, among urban Chinese population; however, a similar study reported even a lower prevalence of AMD in Indian population [9, 10]. There are only few observations on the prevalence of diabetic retinopathy (DR) in Asian population, and the prevalence of retinopathy was higher in Chinese (15%) and Malay population (10%) as compared to Indians (5.3%) [11]. Glaucoma was the main cause of both unilateral and bilateral blindness in Singapore and Mongolian population [12, 13]. The prevalence of glaucoma ranges from 2.15% in Bangladesh to 5.0% in Japanese population [14, 15]. Congenital glaucoma as a cause of blindness is more common in the Middle East Asian countries which can be attributed to high rates of consanguineous marriage [16]. Genetic markers for presymptomatic and prenatal diagnosis are available for specific diseases such as primary open-angle glaucoma and retinoblastoma; however such tests are not being commonly performed in most Asian population.

35.2 Goals of Genetic Counseling

Over the years, the role of genetic counseling in ophthalmology has increased. After the completion of the Human Genome Project and the recent progress achieved in understanding the functionality of genes, their roles have been extraordinary in ophthalmic genetics [17]. With increasing education levels in Asian countries, comprehensive eye care especially involves access to new research findings. By knowing the inheritance pattern of a condition, patients and family members could be helped to make more informed choices, which may hold particular significance for those expecting eventual complete loss of vision. Most molecular diagnostics for genetic eye diseases are examples of presymptomatic diagnosis, which would be of great value for the genetic eye disorders that have available measures of prevention or treatment.

Apart from the routine diagnostic/treatment options which are suggested in a genetic counseling setup, recent advances made in understanding of the molecular basis of eye disease have opened new ventures for personalized or precision ophthalmology [18]. Patients with inherited retinal disease (IRD) pose a compelling case for personalized ophthalmology and demonstrate the need for genetic testing [19]. In a clinical setting, this is being achieved using custom-based target enrichment panels or whole-exome sequencing approaches along with bioinformatics analysis focusing upon genes causing IRD [20]. One such study in 50 patients with IRD has analyzed 105 genes and had been successful in identifying disease-causing mutations in more than 50% of patients [21]. Such study results can be utilized for suggesting genetic testing in the same population for new patients.

35.3 Genetic Counselors and Educators

While advances are being made in genetic research, it is crucial in translating the benefits of medical genetics and genomics to patients and their families. Zayts et al. in their observation from the Asian Pacific Conference on Human Genetics noted that in the Southeast Asian region genetic counseling positions are available in Thailand (for thalassemia only) and Indonesia [22]. In Hong Kong and Malaysia, there are currently no genetic counselor positions supported by the Department of Health (Hong Kong) and the Ministry of Health (Malaysia), respectively. In Hong Kong, the services are provided by a range of professionals including clinical geneticists, obstetricians specializing in - maternal-fetal medicine, registered nurses, oncologists, surgeons, and pathologists. In Malaysia, there were only nine medical geneticists and two associate genetic counselors

providing the genetic counseling service, the ratio of medical geneticists to the total population being 1:3 million. There are only two medical genetics residency programs in India. For a population of over a billion, there are only two centers providing postgraduate courses in genetic counseling in India [23]. According to a report, it is estimated that there are only 50 medical/clinical geneticists in India and about 15 genetic counselors out of which only 16% had undergone a master's training program [24]. Moreover, in most other Asian countries, access to geneticists is inequitable, and there appears to be no networking among them.

A genetic counselor is a health professional who has acquired education and training with a defined curriculum at master's level which is helpful in developing the necessary competence. The aim of training genetic counselors should be that they should have the relevant competence to practice appropriately in terms of appropriate scientific and research training in a relevant setting. While genetic counselors for medical genetics might exist, there is a strong rationale to have dedicated ophthalmic genetic counselors who can cover the various subspecialties within ophthalmology. Genetic counselors need to work in a multidisciplinary environment, and the education and training should reflect the involvement of many disciplines. So there is always a need to assimilate the practical and theoretical contributions from clinical geneticists, specialist medical doctors, primary care physicians, and scientists working in the field of various disciplines such as molecular genetics and cytogenetics. The assimilation can be further enriched by contributions from psychologists, lawyers, ethicists, lawyers, epidemiologists, and sociologists.

The role of genetic counselors is very significant. Genetic counselors should help patients collect, select, interpret, confirm, and analyze information (including family, medical history, pedigree, laboratory results, and literature) relevant to the delivery of genetic counseling for individuals or families. Their role in helping people to understand the psychological, medical, social, and familial implication of ocular genetic disease is important. The education provided about inheritance, management, and prevention and to apply expert knowledge to facilitate the individual or family to access the appropriate healthcare resources, however, is lacking in most Asian countries.

It is clear that training an individual to become a genetic counselor is an expensive and -time-consuming affair. While the long-term goal should be to develop training programs within regions of Asia, can anything be done now to help the ophthalmologist in a busy and overburdened medical system in Asia? Some evidence comes from a work cosponsored by the World Society of Paediatric Ophthalmology and Strabismus (WSPOS) and the University of Pittsburgh, Genetic Counseling Program. In a research-based program, a team from Children's Hospital of Pittsburgh of UPMC Eye Center, 35 optometrists and nurses from nine ophthalmic centers across India, were successfully trained to take a three-generation pedigree without interpretation, prior to the patient(s) seeing the doctor [25]. This increased awareness and diagnosis of IED among doctors and clinic staff alike.

35.4 Process of Genetic Counseling

The awareness of the general ophthalmologists regarding the existence of genetic tests remains low in most Asian countries. On identifying the presence of an inherited eye disease through clinical findings, the prospective value of genetic testing should be discussed with the patient by the treating ophthalmologist, and if any appropriate test (if any) is available, it can be advised. They can also offer a referral to another physician or counselor with expertise for suggesting the selection as well as interpretation of genetic tests. However, certain things have to be kept in consideration that unnecessary parallel testing should be avoided, restrict massively parallel strategies such as whole-exome sequencing and whole-genome sequencing to research studies conducted at tertiary care facilities, testing asymptomatic minors for untreatable disorders except in extraordinary circumstances.

Individuals coming for genetic counseling should be clarified of the purpose of counseling

in the context of accurate risk. This is fundamental in explaining to a patient how the disease is inherited and implication for other relatives. Risk calculation primarily relies on the application of Mendelian genetic principles of inheritance which can be further modified using mathematical principles such as Bayesian analysis for individualized risk assessment [26]. For this, a detailed medical history as captured in a pedigree is the first crucial step which can be used to establish a diagnosis and pattern of inheritance and identify healthy relatives who could benefit from medical screening tests. Other factors that can affect risk assessment depend on whether the genetic condition has variable expressivity, reduced penetrance, and heterogeneity [27]. Genetic testing should always be presented to patients and families as a voluntary procedure and should only be utilized after the patient has been educated about the process. Risk communication plays an important role in making the patients informed of the available choices. So framing of risk should be a powerful way of presenting the risk which could be in a positive or negative manner. Furthermore, it is very important to understand whether the patient and his family have understood the risk information.

Routine follow-up is important in pediatric genetic counseling, as a large amount of information to be shared in a stressful condition could be limited to each visit, and psychological counseling can also be provided. It also provides an opportunity to review the result and clarify the misconceptions that family may have during the process.

35.5 Barriers to Genetic Counseling

The elementary level of genetic training in most Asian countries involves an unstructured and rudimentary medical genetics being taught in undergraduate medical course. Approximately 90% of the medical colleges in India do not provide genetic services or clinical or genetic counseling units which result in physicians not learning genetic/counseling skills and also few opportunities to take up this field as a career option. So the primary care providers may not always be able to make a definitive diagnosis of a genetic disease. There are no guidelines as to when genetic testing should be done, and there are almost no trained genetic counselors or courses to train them. Moreover, in most Asian countries, there are no accreditation bodies or best-practice guidelines for laboratories conducting genetic tests.

Apart from these issues, the major constraints in successful implementation of genetic counseling or tests are public misconceptions, cultural barriers, and traditions. The impact of religion on beliefs regarding congenital birth defects is understood to be the consequences of one's "karma" in a Thai population where majority are Buddhists [22]. In some cultures, mothers are often targeted if the disorder is observed during pregnancy, and it makes it difficult for counseling because they may be abandoned [28]. In many countries where the knowledge of eye disorders is limited, it is a challenge even to get a proper family history. Another problem is the language diversity of the populations in Asian region [29]. As Meenakshi Bhat from the Centre for Human Genetics in Bangalore in India points out, an easily understood word for "gene" or "genetic disorder" is not available in any vernacular language [30]. Perceptive differences to genetic counseling are a major barrier to provide such services in most countries in this region. Asians are less receptive to genetic counseling and are less likely to inform their relatives about their disease [31]. The indications for visiting a genetic counselor also vary in different socioethnic groups. In Iran, it could be a consanguineous marriage that could motivate a couple to seek counseling, while in Pakistan it could be the death or an abnormality in the previous child, for which the couple seek counseling in an already ongoing pregnancy [32, 33].

Most importantly, genetic tests in developing countries are an expensive proposition. Only a small percentage of the population can afford genetic testing and genetic counseling because of the absence of a functional social healthcare system or compulsory health insurance. This could be

attributed to the lack of public funding dedicated to development of genetic services. Apart from the costs involved, most Asians do not want to accept genetic testing as they believe it would not significantly change the clinical outcome [34].

Various ethical issues related to confidentiality and privacy protection are also neglected. Family history, carrier status, and at-risk factor can be stigmatizing especially in settings where the patients and their relatives have low literacy levels. The psychosocial impact of the carrier status is very crucial in most of the Asian societies, and most of the time, the patient is worried about the future personal health risk and change in family dynamics. To communicate effectively the counselor thus must consider the educational, religious, or ethnic background which may lead to directive genetic counseling.

35.6 Future Prospects of Genetic Counseling in Asia

Despite the barriers, the prospects of personalized ophthalmology still look promising as illustrated by the enhanced experiences of some patients with inherited eye diseases. Retinoblastoma mutation testing and its success in preventative strategies have led the WHO to suggest adopting NGS for enhancing genetic tests [35]. New genetic discoveries and the growth of bioinformatics from the Asian countries have heralded a new era in genetics research, which will eventually be taken to the bedside. However, what needs to be shown is the vast economic benefits of genetic research and its application in many developing countries in Asia.

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Epigenetic Study in Asian Eye Diseases 36

Lai Wei, Xiao Hu, and Xiaofeng Wen

Abstract

Understanding the pathogenesis of eye diseases has been significantly advanced by numerous genetic studies in recent years, as well as identifying heritable genetic risk factors and their associated molecular mechanisms for almost every eye disease, such as age-related macular degeneration (AMD) (Fritsche LG, et al., Annu Rev Genomics Hum Genet 15:151–171, 2014) and cataract (Shiels A, Hejtmancik JF, Prog Mol Biol Transl Sci 134:203–218, 2015). However, nongenetic environmental factors have also demonstrated their important roles during development of ocular diseases (Yan B, Yao J, Tao ZF, Jiang Q, J Cell Physiol 229(7):825–833, 2014; Cvekl A, Mitton KP, Heredity (Edinb) 105 (1):135–151, 2010; He S, Li X, Chan N, Hinton, Mol Vis 19:665–674, 2013; Liu MM, Chan CC, Tuo J, Curr Genomics 14(3):166–172, 2013). Despite the significant advances that have been made in understanding the epigenetic regulation in cancer and inflammation, inheritable and dynamic epigenetic changes characterizing ocular diseases are largely unknown (Nickells RW, Merbs SL, Arch Ophthalmol 130(4):508–509, 2012; Cvekl A, Mitton KP, Heredity (Edinb) 105(1):135–151, 2010). In this chapter, we discuss current understanding of the epigenetic regulation and therapies for several most popular eye diseases in Asian countries, namely, cataract, glaucoma, diabetic retinopathy, age-related macular degeneration, retinoblastoma, and uveal melanoma.

Keywords

Epigenetics • Eye diseases • DNA methylation • Histone modification • Epigenetic therapy

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36.1 Twin Studies

The classical way to dissect the genetic versus environmental contributions to complex diseases is the twin study design. Using monozygotic and dizygotic twins, an estimation of genetic heritability, identification of causal genes/mutations/SNPs, and understanding the epigenetic regulation in diseases can all be achieved [8]. As early as in 1922, Walter Jablonski studied heritability of refraction in identical and nonidentical twins, marking the earliest application of twin study design in researches of ocular phenotypes [9]. In recent years, other large identical twin studies have also provided insights into the heritability of ocular traits including intraocular pressure and eye color (Fig. 36.1), as well as eye diseases such as AMD, glaucoma, cataract, and myopia (Fig. 36.2) [8]. Identical twins are often concordant for many ocular traits under physiological conditions (Fig. 36.1) [10–27]. For example, the heritability of eye color, measured by the concordant rate between identical twin pairs, is as high as 98% [16], while a 60% of the average heritability of intraocular pressure is found from five twin studies in various ethnic groups [10–15]. In contrast, the heritability of some ocular diseases is relatively low (Fig. 36.2), including AMD, dry eye disease syndromes, diabetic retinopathy, and chronic open-angle glaucoma [28–38]. This argues that nongenetic factors also play a crucial role in the pathogenesis of these eye diseases.

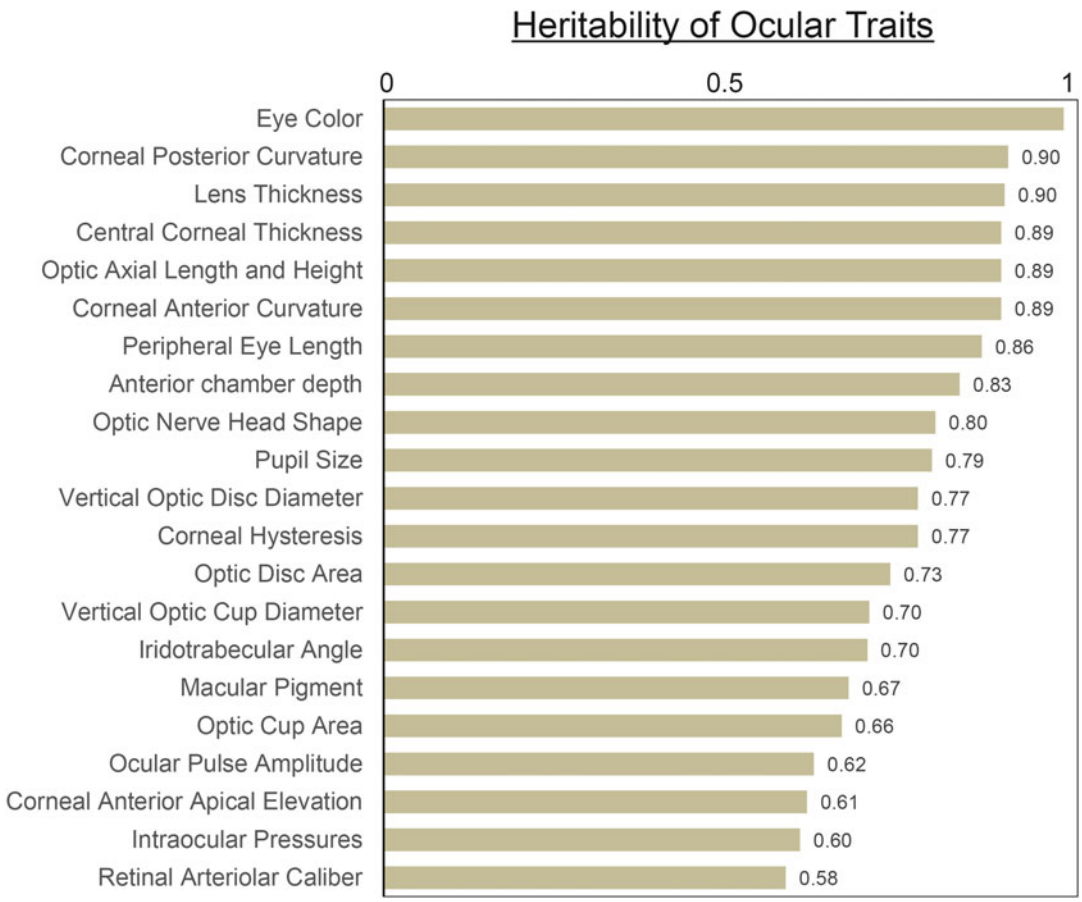

Fig. 36.1 The heritability of ocular traits was decided by the concordance rate of traits between identical twins (Refs. [10–27])

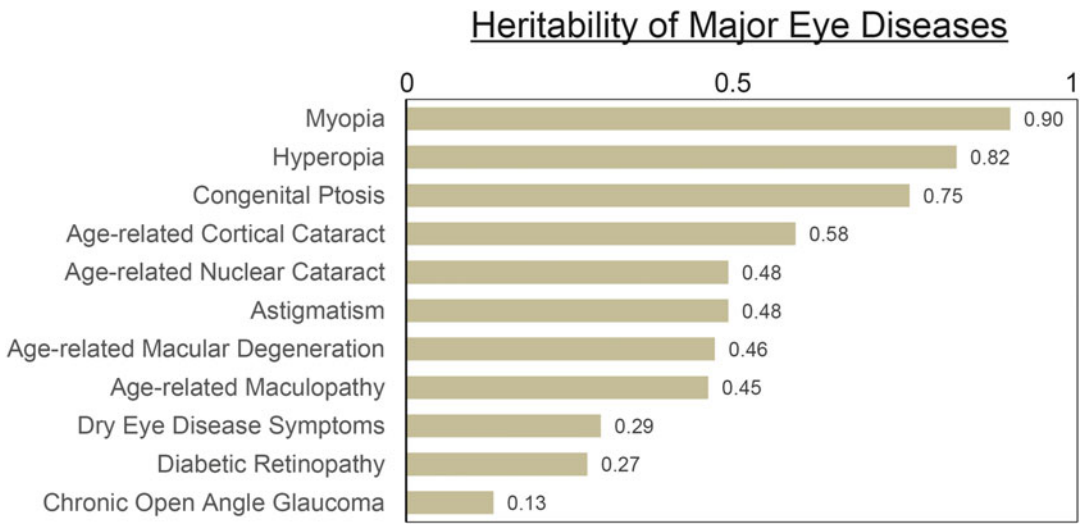

Fig. 36.2 The heritability of ocular diseases was decided by the concordance rate of disease phenotype between identical twins (Refs. [28–38])

36.2 Epigenetics

Epigenetics, first defined by CH Waddington in 1942, is the study of phenotypical changes caused by inheritable and noninheritable environmental influences without changing the DNA sequence [39]. It often refers to the research on the dynamic regulation of transcription by changing the chromatin structure but not underlying DNA sequences. The classical example of epigenetic changes is the cell differentiation, for example, from embryonic stem cells to fully differentiated retinal pigment epithelium (RPE) cell. During this process, many significant gene expression and cellular morphological changes happen without the genomic DNA alterations. All cells in human body also share the almost identical genetic code, while their morphology and function can be diverse. Therefore, it is critical to study epigenetic regulation in eye diseases in addition to genetics.

Epigenetic changes refer to DNA methylation and histone modifications. Numerous proteins including DNA and histone methyltransferases and demethylases, as well as histone acetyltransferases and deacetylases, function to coordinate the machinery of epigenetic regulation that is ultimately responsible for the gene expression regulation [39].

The 5′ methylcytosine is the first form of DNA methylation found in eukaryotes. However, the rate of cytosines that were methylated varies significantly among eukaryotic genomes, ranging from virtually none in yeast and 4% in *Mus musculus* to as high as 14% in *Arabidopsis thaliana* [40]. DNA cytosine methylation occurs predominantly in the symmetric CG context and is generally associated with gene silencing [41]. Actually, about 70–80% of CG dinucleotides are normally methylated in mammalian genomes, called CpG, which tends to cluster to form CpG islands. Many of these CpG islands are located in the regions of gene promoters and demethylated to activate gene expression. They can also be found in the gene bodies of actively transcribed genes in both plant and mammals [42]. In addition, a small amount of non-CG 5′ methylcytosine can be found in embryonic stem cells [43].

In mammalian cells, several genes are responsible for maintaining and dynamic regulation of DNA methylation. DNA methyltransferase 1 (DNMT1) maintains heritable DNA methylation patterns through every cellular DNA replication cycle. This is important to preserve cell type-specific gene expression during proliferation and prevents the passive DNA demethylation in daughter cells. On the other hand, DNMT3a/3b and their cofactors DNMT3L establish de novo

DNA methylation patterns during cell development and differentiation, coordinating the dynamic regulation of gene transcription together with the methyl-CpG-binding proteins (MBDs) and transcription factors in "reading" methylation marks [44].

Although DNA methylation has been extensively investigated, it is until recent years that the molecular mechanism underlying DNA demethylation process has been revealed [45]. DNA demethylation can be passive (likely global) or active (likely locus-specific) processes. While a direct removal of 5-methylcytosine from DNA has not been discovered, a process combining deamination and oxidation of 5-methylcytosine to remove 5-methylcytosine from DNA has been recently defined. In mammalian cells, the ten-eleven translocation proteins (TET1, TET2, TET3), a family of iron-dependent oxygenases, oxidize the methyl group at position 5 of 5-methylcytosine to form 5-hydroxymethylcytosine [46, 47] and even further oxidized forms of 5-formylcytosine and 5-carboxylcytosine [48, 49] to achieve the active conversation of methylated to unmethylated DNA [50].

DNA methylation is an important regulator of transcription in response to environmental cues in physiological process. Aberrant DNA methylation patterns have broad effects on pathogenesis of many diseases such as cancer and other ocular manifestations [51]. Hypermethylation of tumor suppressor gene promoters and hypomethylation of oncogene loci are found in many human malignancies [52]. Global hypomethylation in monocytes and lymphocytes has been found in atherosclerotic patients and can be used as an early biomarker predicting the risk of disease onsite [53]. In addition, a global loss of DNA methylation also contributes to aging process, providing an evidence of biological epigenetic clock [54].

As important as DNA methylation, posttranslational covalent modifications of histones also play key roles in epigenetic regulation of all cellular processes and are often referred to as the histone code [55]. A huge catalog of more than 100 types of histone modification has been found in physiological and pathological conditions in various organisms, including the lysine methylation, lysine acetylation, lysine ubiquitination, lysine sumoylation, arginine methylation, arginine citrullination, serine/threonine/tyrosine phosphorylation, etc. In addition, the histone methylation can also happen in the type of mono-, di-, or trimethylation. Although the functional consequences of most of these histone modifications have not been fully defined, many of the well-defined transcriptional active marks such as H3K4me3, H3K14Ac, H3K9Ac, H3K27Ac, and H3K36me3, as well as repressive marks H3K27me3 and H3K9me3, have been extensively mapped genome widely in numerous cell types and in disease conditions [56].

As histone modification often exists as a complicated pattern, a number of proteins are required in the addition and removal of histone modifications. In particular, those enzymes and proteins mediating histone acetylation and methylation, namely, histone acetyltransferases (HAT) and their inhibitors (HDACs), as well as histone methyltransferases and demethylases, have been extensively studied. They also serve as the drug targets in many diseases such as cancer [57].

Recently, noncoding RNA is found to be crucial in epigenetically regulating the transcription of many genes. Short-interfering RNA (siRNA), microRNA, and long noncoding RNA (lncRNA) are all transcripts whose major roles during lifespan are to interact with the transcription machinery in order to modulate the gene expression. These regulatory RNAs significantly enriched the tool sets using which the cell is able to optimize the best outcome of gene regulation. Although more efforts are needed to elucidate how regulatory RNA functions, they already serve as the biomarkers for many diseases and become the natural therapeutic targets due to relatively easy detection and manipulation [58].

The outcome of transcriptional regulation, either activation or repression of gene expression, results from the collective effects of DNA methylation, histone modifications, and noncoding RNA regulation. In particular, a cross talk between DNA methylation and histone modifications in coordinating the epigenetic regulation

of various cellular functions has been well demonstrated in recent studies [59]. Together, all epigenetic changes decide the chromatin structure and DNA accessibility, contributing to an interconnected dynamic network of transcription regulation in physiological or pathological conditions.

36.3 Cataract

Cataract is a disease of cloudy lens in the eye that decrease vision. As elucidated by the twin studies, both genetic and environmental factors decide the risk of cataract. However, there are very few studies focusing on the epigenetic mechanism underlying the pathogenesis of cataract. Wang et al. found that hypermethylation of the CpG island regulating 8-oxoguanine DNA glycosylase I (OGG1) resulted in reduction of its gene expression lens cortex of age-related cataract patients [60]. Similarly, hypermethylation of CRYAA gene promoter led to decreased expression of CRYAA in lens epithelia of age-related cataract patients, which could be reversed by the DNA methylation inhibitor zebularine [61, 62]. Zebularine as well as HDAC inhibitors trichostatin-A (TSA) and vorinostat were also found to suppress TGF-β-induced epithelia-mesenchymal transition of lens epithelium [63, 64]. In addition, blocking chromatin remodeling factor Brg1 induced cataract in mouse [5]. Taken together, epigenetically regulating gene expression can modulate the pathogenesis of cataract.

36.4 Glaucoma

Glaucoma is a collection of a group of eye disorders leading to the damage of optic nerve, either associated with or without high intraocular pressure. Among all diseases investigated using identical twin cohorts, glaucoma showed the lowest genetic heritability (0.13) (Fig. 36.2). Multiple environmental risks have been suggested to increase the susceptibility of glaucoma. However, it is still unclear which one might be a dominant causal factor for onsite of the disease. The overall DNA methylation level was found different in peripheral blood mononuclear cells in open-angle glaucoma patients as compared to controls, although the detail mechanisms by which aberrant DNA methylation may contribute to the etiology of glaucoma is not known. Recent studies suggested that HDAC inhibitor TSA and valproic acid can also reduce the loss of ganglion cells after glaucoma-like tissue damage, suggesting that both DNA methylation and histone acetylation could be potential molecular events targeted by glaucoma treatment [65].

36.5 Diabetic Retinopathy

Diabetic retinopathy (DR) is the major complication of diabetes. 95% patients with over 20 years of diabetes and 80% patients with type II diabetes have the signs of DR [66]. Chronic elevation of blood glucose and associated inflammation increase the permeability and blockage of retinal blood vessels, leading to microaneurysms, macular edema, and neovascularization, sequentially. Hyperglycemia induces cellular damage through formation of glycation end products and activation of the aldose-reductase metabolic pathway, protein kinase C, and ROS, which is an epigenetic regulatory process [67]. Zhong et al. reported a significant decrease in H3K4me2 that was found at the promoter of Sod2 gene, resulting in compromised expression of Sod2 retinal tissues from DR patients. Sod2 protects retinal endothelial cells from glucose-induced oxidative stress. Therefore, epigenetically regulating its expression may serve as partially the molecular mechanism underlying DR pathogenesis [68]. Furthermore, the matrix metalloproteinase 9 (MMP9) gene, which can induce mitochondria damages and retinal capillary cell death, is also epigenetically regulated in DR patients. Elevated promoter H4K9Ac was found on the promoter of MMP9 gene in the retina of both DR patients and rat DR models [69]. In addition, Tewari et al. showed that hypermethylation of DNA polymerase gamma (POLG1) gene promoter as well as increase of

DNMT activity in the retina may all promote the progression of DR [70]. Taken together, it is essential to fully understand the epigenetic gene regulatory networks in order to elucidate the molecular etiology of complex diseases such as DR.

36.6 Age-Related Macular Degeneration

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in elderly worldwide. It leads to progressive loss of central vision due to geographic atrophy (dry AMD) or choroidal neovascularization (wet AMD). Retinal pigment epithelium (RPE) atrophy, photoreceptor degeneration, drusen formation (dry AMD), or pathological angiogenesis and/or hemorrhage in the choroidal macular regions are major hallmarks of AMD. Genetic and genomic approaches have identified many susceptible genes and provided important insights into the pathogenesis of AMD. Currently, in addition to the definition of neurodegenerative disease, AMD has been also defined as an inflammatory disease due to identification of complement factor H (CFH), CFB, C2, C3, and CFI, ARMS2 and HTRA1, tissue inhibitor of metalloproteinase 3 (TIMP3), HLA, and interleukin 8 (IL8) as strong genetic contributors to AMD progression [1].

Both genetic and environmental factors contribute to the development of AMD. Several behavioral and nutritional factors have been suggested to be associated with AMD pathology. Heavy smokers have higher risk of AMD, especially more advanced stage of AMD and larger drusen size than nonsmokers, while higher intake of fish, omega-3 fatty acid, dietary vitamin D, betaine, and methionine is beneficial for preventing AMD. Further studies are needed to identify a full spectrum of environmental factors associated with AMD etiology [71–74].

Recent studies started to tackle the molecular epigenetic mechanism underlying AMD pathology. Genome-wide DNA methylation analysis identified 231 genes whose promoters were differentially methylated between three pairs of twins with discordant advanced AMD phenotype. The hypomethylation of *IL17RC* promoter, which results in the elevated expression of the IL-17RC protein on monocytes in peripheral blood and retinal tissues of AMD patients, suggesting that epigenetic regulation of inflammatory gene *IL17RC*, may play an important role in AMD [75]. Interestingly, interleukin 17 (IL-17) and IL-22, produced by a subset of $CD4^+$ Th17 cells in the serum of AMD patients [76], can induce demethylation of the *IL17RC* promoter and promote *IL17RC* expression in peripheral blood and retinal tissues of AMD patients [75]. Hunter et al. compared genome-wide DNA methylation patterns between post-mortem choroidal tissues from AMD patients and controls and found hypermethylated promoters of glutathione S-transferase isoform mu1 (GSTM1) and GSTM5 genes and reduction in their protein expression in AMD patients [74]. Promoter hypomethylation of clusterin, a major component of drusen deposits in RPE cells from AMD patients, has been also found [77]. Moreover, the expressions of enzymes involved in epigenetic regulation such as HDAC1, HDAC3, HDAC6, DNMT1, and DNMT3a were all altered in RPE cells from mice with AMD-like retinal degeneration [67]. Taken together, epigenetic regulation in AMD is a crucial way during AMD development.

36.7 Retinoblastoma and Uveal Melanoma

Retinoblastoma is the most common childhood ocular malignancy. Mutations in the cell-cycle-control gene RB1 have been considered as the leading cause of retinoblastoma [78]. However, multiple tumor suppressor gene promoters, including RB1 itself, are found hypermethylated in retinoblastoma [79]. This phenomenon initiated numerous studies investigating whether promoter methylation of tumor suppressor genes may drive tumorigenesis. On the other hand, uveal melanoma is a common ocular

tumor in adults. Similar to the retinoblastoma, hypermethylation of the RAS association domain family 1A gene (RASSF1A) was also found in uveal melanoma [80].

36.8 The Future: Epigenetic Therapy for Eye Diseases

In the past decade, a number of epigenetic abnormalities have been found to contribute to the development of many human diseases. Our growing understanding of how human diseases are linked to epigenetic changes has led to novel therapies for diseases such as cancer, inflammatory diseases, neuropsychiatric, and metabolic disorders [52, 57]. US FDA has approved several DNA methylation inhibitors, including 5-azanucleosides, azacitidine, and decitabine, as well as histone deacetylase inhibitors such as vorinostat and romidepsin for cancer therapy. Other small molecules targeting the HDAC and histone methyltransferases are under development and clinical trials for treatment of various cancers [57].

A growing body of evidence indicates critical role of epigenetic regulation in eye diseases [5, 6]. Therefore, targeting the epigenetic regulators is a promising new approach for treatment of these ocular manifestations. TSA and HDAC inhibitors potentially are able to suppress inflammation and angiogenesis, protect retina from ischemic damage, and help to restore visual acuity. They are under extensive investigation for controlling AMD, DR, cataract, and glaucoma in addition to ocular and orbital tumors [67].

36.9 Conclusion

Epigenetic regulatory mechanism has been extensively investigated in many physiological processes of human body and some diseases such as cancer and inflammatory diseases. However, how eye diseases are controlled epigenetically is largely unknown. In fact, most of the current therapeutic drugs could not directly change the DNA sequence or genetic code nature has set up [52, 81]. However, one can rather aim to correct the environment inside and outside of the body, which adjusts the epigenome of cells and restore the physiological function in patients. Therefore, the future medicine for eye diseases is eye on epigenetic therapy.

Compliance with Ethical Requirements The authors have no conflict of interest to declare.

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Unique Genetic Signatures in Asian Age-Related Macular Degeneration: An Opportunity for Drug Development

37

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Abstract

Genetics plays a major role in the pathogenesis of age-related macular degenerations (AMD). An estimated 75% of AMD cases worldwide involve genetic variation. Although AMD is common in both Asian and Western populations, there are differences in pathological phenotypes in Asians compared to Western world and response to treatment (e.g., standard anti-VEGF therapy does not provide favorable outcome for Asian patients). Polypoidal choroidal vasculopathy (PCV), a variant of AMD, accounts for a much larger proportion of AMD in Asian countries. Further, emerging studies are showing the association of new specific loci for East Asian AMD and PCV patients compared to Western populations. How this information can be used for future risk prediction and drug development that could be specifically targeted to Asian population is discussed in this chapter.

Keywords

Macular degeneration • Polypoidal choroidal vasculopathy • Neovascularization • Genetics • Asians • Risk factors • Genetics • Anti vascular endothelial growth factor

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37.1 Background

Whether it's the nature (genetics) or nurture (environment) that plays a role in developing any disease risk factors is a long-standing debate. Further, individual choices add to the complexity to this debate. It has been established that the prevalence of not only eye diseases [1] but several other chronic diseases such as cardiovascular and diabetic diseases [2] is increased due to individual choices and lifestyles. Therefore, it has become critical to identify risk factors for any diseases in subset of populations belonging to specific ethnic community. Here, we discuss the significance of specific genetic markers in Asian population and their risk in developing age-related macular degeneration (AMD) and how this information could be useful in risk prediction and in designing novel treatment therapy that is targeted toward Asian population.

37.2 Age-Related Macular Degeneration (AMD)

AMD is a leading cause of central vision loss in patients older than 65 years of age [3]. The disease attacks the macula of the eye, where the sharpest central vision exists, needed for daily tasks such as reading, driving, and recognizing faces. AMD is a complex and multifactorial disorder (Fig. 37.1), and the prevalence of the disorder is increasing with increased longevity around the world affecting almost 1 in 4 people by age 80 [4]. The projected number of people with AMD in 2020 is estimated ~196 million [5]. There are two types of AMD, the dry and wet forms. Dry AMD is a more common disease that causes some degree of visual impairment and sometimes progresses to blindness. In wet AMD (~10–15% of AMD cases [6]), patients experience abnormal neovascularization, which originates from the choroid (hence, referred to as choroidal neovascularization (CNV)) and rapidly destroys photoreceptors and vision. CNV is induced largely by vascular endothelial growth factor A (VEGF-A) [7–9]. This growth factor, with a central role in both normal and pathologic vascular growth within the eye [9–13], binds to VEGF-A receptors (e.g., Flt-1) on the vascular endothelium. Despite success with anti-VEGF therapy (Avastin®, Lucentis®, Eylea®), a significant number of wet AMD patients do not respond with appreciable clinical improvement

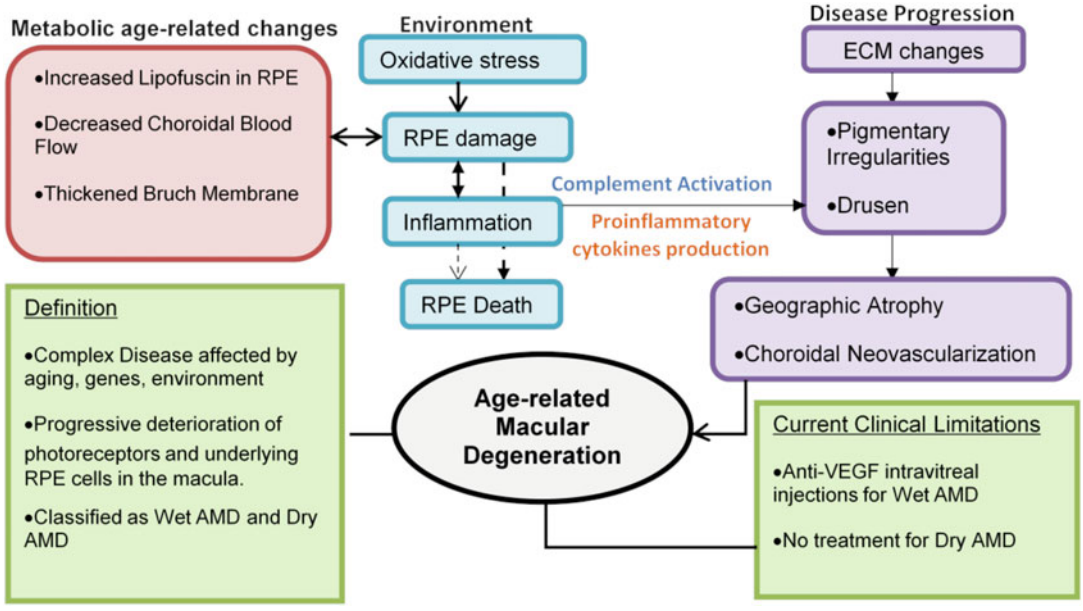

Fig. 37.1 Brief overview of age-related macular degeneration (Courtesy of Veluchamy A Barathi; Su Xinyi and Tay Yong Ren, Singapore Eye Research Institute)

[14, 15]. In fact, a recent study has revealed that 7-year mean visual outcomes actually drop below baseline visual acuity [16, 17] with anti-VEGF therapy [18]. This long-term decline in the efficacy of intravitreal anti-VEGF therapy is concurrent with substantial rates of sub retinal fibrosis (61% at 7 years) and geographic atrophy (90% at 7 years) [17, 19, 20]. Recent findings from follow-up of the CATT cohorts showed 45.3% of patients developing scars at 2 years, of which more than half were classified as "fibrotic," with solid elevation disrupting retinal architecture [21]. The nonresponsiveness of AMD patients and insufficient efficacy of anti-VEGF drugs are not due to their inability to block VEGF pathway but rather involvement of VEGF-independent angiogenic pathways involved in the abnormal neovascularization, and therefore anti-VEGF therapies alone are not sufficient to treat AMD. Further, it has been demonstrated that multiple intravitreal injections of anti-VEGF agents induce RPE degeneration in mice [21].

37.3 AMD Genetics

It was believed that higher prevalence of AMD is found in Europeans than in Asians, but recent studies show that AMD is as common in Asian populations as in the Western populations [22]. In addition, a variant of AMD called polypoidal choroidal vasculopathy (PCV) is much more common in Asian AMD patients compared to Western population [23]. PCV is a disease of the choroidal vasculature, and it differs from AMD in clinical features and does not response well to the current anti-VEGF treatments. Multiple genetic variants contribute to AMD risk [24]. The risk of developing AMD is threefold higher for someone who has a family member with AMD than the one without [25]. Several genome-wide association studies (GWAS) have identified common variants associated with AMD in Caucasian populations [24, 26–28], and recently, an AMD Gene Consortium consisting of 18 centers worldwide discovered seven new loci that are associated with increased risk of AMD [24]. Increased risk of AMD is associated with single nucleotide polymorphisms in the complement factor H gene on chromosome 1q31 and frequent polymorphism in ARMS2/HTRA1 on chromosome 10q26 [24, 29–33]. Taken together, these two loci account for more than 50% of AMD pathology. Risk alleles in CFH, ARMS2/HTRA1, and cigarette smoking taken together account for approximately 76% of the population-attributable risk for the development of AMD. Unfortunately, smoking is still common in most Eastern countries. CFH polymorphism is more common in Caucasian population; however, virtually every ethnic population has shown consistent associations of ARMS2/HTRA1 locus with AMD. Thus, the new drug development focused on ARMS2/HTRA1 locus would be more effective among Eastern as well as Western countries.

37.4 Genetic Variations in Asian AMD and PCV Patients

In Asian populations, such as in Japanese and Chinese, the clinical features of AMD appear different from the Western populations [34–39], such as the absence of drusen and minimal fibrous scaring in PCV and lesser geographic atrophy and a poorer response to inhibitors of VEFG, compared to those of European ancestry [39, 40]. Figure 37.2a, b illustrates a case of typical AMD (tAMD). A classic appearance of a sea fanlike network due to CNV can be seen in fluorescence angiography. On the other hand, PCV appears as reddish, orange, bulging polyp-like dilations [35] with submacular hemorrhage in the fundus (Fig. 37.2c, d). They arise from the inner choroidal vascular network [34] and are associated with recurrent serous or hemorrhagic detachments of the RPE, vitreous hemorrhage, and minimal fibrous scarring, as compared with tAMD [38]. In most of the studies in Western populations, PCV is estimated to only make up 5–10% of neovascular AMD [36, 37]. In contrast, the prevalence of PCV in Asians is remarkably high. It has been reported to account for 50–60% of all cases of neovascular AMD in Japanese and Singaporeans [37, 39]. While both

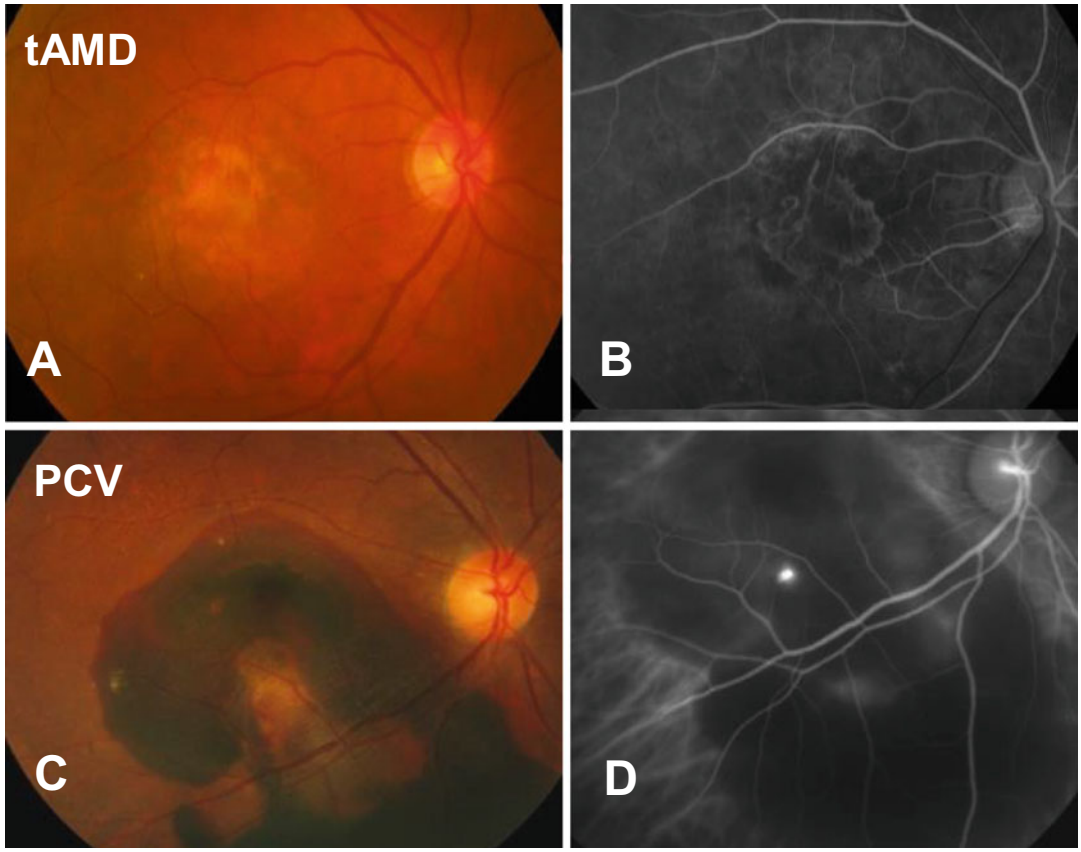

Fig. 37.2 Fundus imaging of AMD vs. PCV. (**A**) Typical neovascular AMD (tAMD) fundus photo and (**B**) fluorescence angiography, showing choroidal neovascularization. (**C**) Polypoidal choroidal vasculopathy (PCV) fundus photo and (**D**) indocyanine green angiography, showing clusters of polyps

PCV and tAMD are considered vasculopathies arising from the choroidal vasculature, controversy remains as whether PCV is a subgroup of AMD or is a separate disease entity. Moreover, PCV affects younger individuals and more likely to complicate massive submacular hemorrhage and even breakthrough vitreous hemorrhage, which again are more uncommon in AMD [39, 41]. These disparities also suggest that susceptibility genes for PCV may be different between Asian and European-ancestry patients and underlying genetic mechanisms of PCV may be different from tAMD.

Common genetic variants for AMD have been identified in populations of European ancestry, [24, 26–28, 42]. In addition, rare genetic variations at CFH, CFI, C3, and C9 were also shown to strongly associate with AMD in Europeans [43–46]. However, there are not too many population-based studies done in Asian AMD patients except recent GWAS and exome-wide association study (EWAS) in more than 6000 East Asian AMD patients [47, 48]. Recently, the Genetics of AMD in Asians (GAMA) Consortium compared the associations of AMD loci identified in Europeans with those in East Asians (who had neovascular AMD). Out of 20 previously reported genetic variants showing strong evidence of association in Europeans, the consortium was able to replicate 9 of them ($P < 0.05$) in East Asians [47] (Table 37.1). Further, there are substantial differences in minor allele frequency (MAF) for most of the previously identified AMD variants between Europeans and East Asians. Using both genome-wide genotype and exome arrays, the GAMA consortium identified three novel loci (C6orf233, SLC44A4, and FGD6) and one novel variant in CETP (Asp442Gly,

Table 37.1 Comparison of the associations of AMD loci reported in Europeans with those in East Asians in the Genetics of AMD in Asians (GAMA) Consortium

SNP	Chr	Locus[a]	MA	East Asians[65] in GAMA			Previous studies in Europeans		
				MAF	OR	*P value*	MAF	OR	*P* value
rs10737680	**1**	***CFH***	C	0.39	0.59	7.5×10^{-38}	0.36	0.41	1×10^{-434}
rs6795735[b]	**3**	***ADAMTS9***	G	0.25	0.81	1.1×10^{-5}	0.54	0.91	5×10^{-9}
rs13081855[b]	3	*COL8A1-FILIP1L*	A	0.04	1.0	0.99	0.10	1.23	4×10^{-13}
rs4698775	**4**	***CFI***	C	0.20	1.17	7.5×10^{-4}	0.31	1.14	7×10^{-11}
rs141853578[b]	4	*CFI*	A	0.00	–	–	<0.01	22.2	4×10^{-6}
rs34882957[b]	5	*C9*	A	0.00	–	–	<0.01	2.20	6×10^{-7}
rs3130783[b]	6	*IER3-DDR1*	G	0.10	1.12	0.06	0.21	0.86	3×10^{-11}
rs429608	**6**	***C2-CFB***	A	0.07	0.74	1.1×10^{-4}	0.14	0.57	4×10^{-89}
rs943080	**6**	***VEGF-A***	G	0.24	0.91	0.04	0.49	0.87	9×10^{-16}
rs3812111[b]	6	*COL10A1*	A	0.18	1.08	0.16	0.36	0.91	2×10^{-8}
rs1999930[b]	6	*FRK-COL10A1*	A	<0.01	1.04	0.94	0.30	0.87	1×10^{-8}
rs334353	**9**	***TGFBR1***	C	0.44	0.91	0.01	0.27	0.88	3×10^{-11}
rs10490924	**10**	***ARMS2-HTRA1***	A	0.47	2.42	1.2×10^{-103}	0.30	2.76	4×10^{-540}
rs8017304	14	*RAD51B*	A	0.45	0.99	0.82	0.61	1.11	9×10^{-11}
rs920915	15	*LIPC*	G	0.22	0.84	0.70	0.52	0.88	3×10^{-11}
rs3764261	**16**	***CETP***	A	0.17	1.41	1.7×10^{-12}	0.33	1.15	7×10^{-9}
rs2230199[b]	19	*C3*	C	<0.01	2.45	0.19	0.20	1.42	1×10^{-41}
rs147859257[b]	19	*C3*	C	0.00	–	–	<0.01	3.13	7.1×10^{-7}
rs4420638	**19**	***APOE***	G	0.10	0.86	0.03	0.17	0.77	2×10^{-20}
rs8135665	22	*SLC16A8*	A	0.14	1.08	0.16	0.21	1.15	2×10^{-11}

[a]A locus is defined as a region flanking up to 250Kb of a genome-wide significant SNP
[b]Including Yu Y et al. Hum Mol Genet (2011), Fritsche LG et al. Nat Genet (2013), Seddon JM et al., Nat Genet (2013), Helgason H et al., Nat Genet (2013), and Zhan X et al., Nat Genet (2013)

rs2303790), significantly associated with neovascular AMD in Asian [47]. Furthermore, a recent whole-exome sequencing (WES) study identified UBE3D as a novel gene for neovascular AMD in East Asians [49]. Investigations into PCV genetics have been focused on candidate gene studies. *CFH* variants rs3753394 (odds ratio [OR] = 2.1) and rs800292 (OR = 2.0) were significantly associated with PCV [50], while the *CFH* Y204H variant (identified through GWA studies of AMD) was not. Genetic variants in *HTRA1* associated with PCV (rs11200638, OR = 2.2) with an effect size similar to AMD [50], yet OR estimated from other studies for tAMD was about twice as high as that for PCV. In contrast, other complement-related genes, such as *C2* variants appear not associated with PCV [50]. A recent meta-analysis study found that 25 polymorphisms in 13 AMD-associated genes (*ARMS2, HTRA1, C2, CFB, ELN, LIPC, LPL, ABCA1, VEGF-A, TLR3, LOXL1, SERPING1, and PEDF*) had no significant association with PCV [51]. Taken together, these findings suggest genetic architectures of AMD in Asians could be distinct from those in Europeans. Further studies in Asians, either of the larger sample size or using next-generation sequencing technologies, would be needed to identify AMD risk variants with smaller effect sizes and/or lower minor allele frequencies. Since Asians are highly heterogeneous, studies on non-East Asians, such as South Asians, are also needed to have a more comprehensive understanding of genetic characteristics of AMD in Asians.

37.5 New Genome Sequencing Technologies in Genetic Variant Discovery

Since the first GWAS of AMD reported in 2004, several AMD loci and variants have been discovered through such approaches. Although GWAS is powerful in identifying common variants (defined as minor allele frequency >5%), its

ability and power in discovering rare disease variants is somewhat limited. In addition to common variants, it is important to identify low-frequency (1% to <5%) and rare (<1%) disease variants, as they are more likely to be functional and have considerably higher genotypic relative risk than common variants involved in complex disease etiology. Recent next-generation sequencing platforms have dramatically improved sequencing efficiency, allowing us to investigate rare variants using target sequencing, whole-exome sequencing (WES), and/or whole-genome sequencing (WGS) technologies. Using next-generation sequencing, studies have identified rare variants at CFH, CFI, C3, and C9 associated with AMD risk [43–46]. It is anticipated that pathogenic variants that are amenable to functional genomic modeling and drug discovery efforts will be identified via next-generation sequencing in the near future. Low-pass WGS has been proven as an effective approach to maximize the effective sample size in population-based genetic disease studies with a limited budget [50, 52]. Through the 1000 Genomes Project, which has sequenced ~2500 individuals from 26 diverse populations at ~4X genome-wide, a series of bioinformatics tools have been developed to accurately identify genotypes from low-pass WGS. These genotype algorithms leverage the haplotype information shared by multiple samples included in the sequencing experiments to improve genotype calling at each variant site. With these newly developed algorithms, almost all variants with minor allele frequency >1% can be discovered and genotyped with high accuracy (~100% discovery rate and >99.9% accuracy across all sites and >97.3% accuracy at heterozygous sites) [50]. To better dissect the genetic basis of PCV in Asia, especially the role of low-frequency variants in both coding and regulatory noncoding regions across the genome, a WGS study can be performed using limited number of controls and to supplement the sample size with a large number of general controls whose GWAS or WGS data are available. This strategy of using general controls to increase statistical power has been used in many genetic association studies, including a recent targeted sequencing study of AMD [46].

37.6 Potential AMD Targets Through Genetic Studies

The current anti-VEGF treatment for AMD is not universally effective as large number of patients do not response to the therapy suggesting that "one size fit all" model does not work for AMD and there is need to investigate why some patient population do not respond to the therapy. Further, clinical phenotypes in Asians AMD patients are different from Caucasian populations. For example, in PCV, drusens are absent, and there is minimal fibrosis/scarring compared to Caucasian AMD patients [39]. In addition, outcomes of anti-VEGF therapy are not favorable in PCV patients [40]. There is a lack of PCV-specific treatment due to poor understanding of the underlying pathogenesis. In PCV, additional pathways likely play a more important role. For example, it is known that the branching vascular network continues to enlarge despite anti-VEGF therapy or PDT and may lead to chronic exudation and polyp recurrence. Thus, novel therapeutic targets specific for PCV are urgently needed. The search for newer molecular pathways through genetic studies using Asian population remains highly relevant from both clinical and industry perspectives for the treatment and prevention of PCV. Human Genome Project has provided valuable "pharmacogenomic" information on how inherited gene variations affect one's response to medications. Further, the individual genetic differences could also be useful to develop therapy that will be effective for a particular person or specific ethnic population. Therefore, it is critical to learn the genetic variations that are specific in Asian AMD populations. Although the previously identified 19 loci associated with AMD [24] were replicated in the Asian populations, new loci specific to Asian AMD patients were identified including Asian-specific mutation in CETP (cholesteryl ester transfer protein) involved in the transfer of cholesteryl ester from high-density lipoprotein (HDL) to other lipoproteins [47, 48]. The CETP Asp442Gly (rs2303790) mutation increased AMD risk (odds ratio: 1.70) in Asian patients. In addition, three novel loci

were identified. These are C6orf223 Ala231Ala, SLC44A4 Asp47Val, and FGD6 Gln257Arg [47]. SLC44A4 and FGD6 are non-synonymous variants. C6orf223 is a newly mapped gene with yet unknown functional role. SLC44A4 encodes for choline transporter protein-4, involved in sodium-dependent choline uptake by cholinergic neurons. Defects in SLC44A4 have been linked to lysosomal storage disease which presents wide spectrum of symptoms including eye abnormalities. FGD6 encodes FYVE, RhoGEF, and PH domain-containing protein 6, with unknown function. Interestingly, several studies have also found CETP association with PCV risk, suggesting lipid pathways are affected in PCV [53, 54].

37.7 New Paradigms in Drug Discovery

There is an enormous interest from pharmaceutical sector to take advantage of the genetic information to identify new drug candidate and develop novel therapy. This has been made possible by the affordability of genome sequencing compared to just few years ago. Entire human genome can be sequenced today in less than $1000.00 compared to billions of dollars just a decade ago. Any rare genetic changes in human genome can be compared with ease by scanning large databases of genomic sequences to validate the mutations to disease. Regeneron Pharmaceuticals is collaborating with Pennsylvania's Geisinger Health System to sequence genomes of close to 250,000 volunteers and is already claiming discoveries based on the new approach. They claim that they have identified several genes that could prove novel drug targets for various diseases. Similarly, Pfizer, Roche, and Biogen are using human genome sequence database to find new drug targets. Genetic sequences would also be helpful in identifying biomarkers, designing clinical trials, determining individual's drug response, and facilitating combination therapies.

37.8 Drug Discovery Using Genes Associated with PCV

Genes/loci that are associated with PCV susceptibility include ARMS2, HTRA1, CFH, C2, CFB, RDBP, SKIV2, and CETP [51, 55]. These genes are related to extracellular matrix, basement membrane, complement cascade, lipid metabolism, and inflammation, suggesting that cellular damage induced by inflammation contributes to the pathogenesis of PCV. Previous studies, based on the finding of associated genes, have demonstrated that drusens are the product of local inflammation resulting from RPE disorders involving the immune system and the complement component C3 contained in the drusen of patients with AMD [56]. The activation of C3 through the alternative pathway leads to the formation of the membrane attack complex C5b-9, which induces dysfunction of the RPE cells.

Recently, transgenic mice overexpressing human HTRA1 in the RPE were demonstrated to show PCV-like capillary structures in the choroid [57, 58] suggesting that overexpression of HTRA1 alone can evoke choroidal vasculopathy or neovascularization observed in PCV. The protein encoded by CETP gene is found in plasma and involved in the transfer of cholesteryl ester from high-density lipoprotein (HDL) to other lipoproteins. The Asp442Gly mutation results in abnormal protein and is linked to hyperalphalipoproteinemia 1 (HALP1) disease [59]. The association of Asp442Gly (rs2303790) in AMD is the strongest outside the two most common variations found on chromosome 1 and 10. The Asp442Gly mutation is seen only in East Asians (e.g., Chinese, Japanese, and Koreans) and not in South Asians, Europeans, or Africans [47]. This mutation is independent from all previously described polymorphisms near the CETP locus. Earlier studies have shown that dysregulation of lipid metabolism pathway is involved in AMD [53, 54] suggesting that dietary fat intake could play some role in developing the risk for AMD. Retinal pigment epithelial (RPE) cells represent a primary site of pathology in AMD and their

function and phenotype is compromised in AMD. It is possible that CETP mutation may cause drusen formation and alterations in RPE and Bruch's membrane that ultimately induces AMD pathogenesis. However, before any of the abovementioned genes can be validated as a novel drug targets for AMD, it is important to address the following key questions:

1. What is the effect of gene variations on early RPE injury markers such as drusen formation, changes in RPE function affecting transepithelial resistance (TER), and cell adhesion and migration?
2. What is the mechanism by which genetic variations cause AMD pathogenesis?
3. Most importantly, using transgenic mice expressing the genetic variations, is it possible to demonstrate vulnerability of these mice in developing phenotypes of AMD/PCV?
4. Last, is there any effect of genetic variations on the regulation of any known genes/proteins involved in AMD/PCV pathogenesis?

To answer the above questions, one needs the relevant in vitro as well as in vivo models to understand the role of genetic variations on AMD/PCV pathogenesis. Some of these studies can be easily performed in the lab by making various combinations of expression constructs and determining their effect on RPE function using various cell-based assays to characterize drusen formation, effect on retina blood barrier function, and retinal endothelial migrations. Yeast two-hybrid system can be used to compare the interaction of wild-type and mutant target gene with other proteins in lipid pathway and proteomics to investigate any differential regulation of known proteins in the retinal cells expressing wild-type vs. mutant gene. Detailed investigations using abovementioned assays and studies could identify new drug candidates and also provide new avenues for developing drug screening platforms that are more relevant to actual human disease. A schematic flow diagram for drug discovery using genetic signatures is shown in Fig. 37.3.

37.9 Animal Model

Animal models are necessary for drug discovery so that candidate drug molecules can be screened to establish the efficacy of the new molecules as

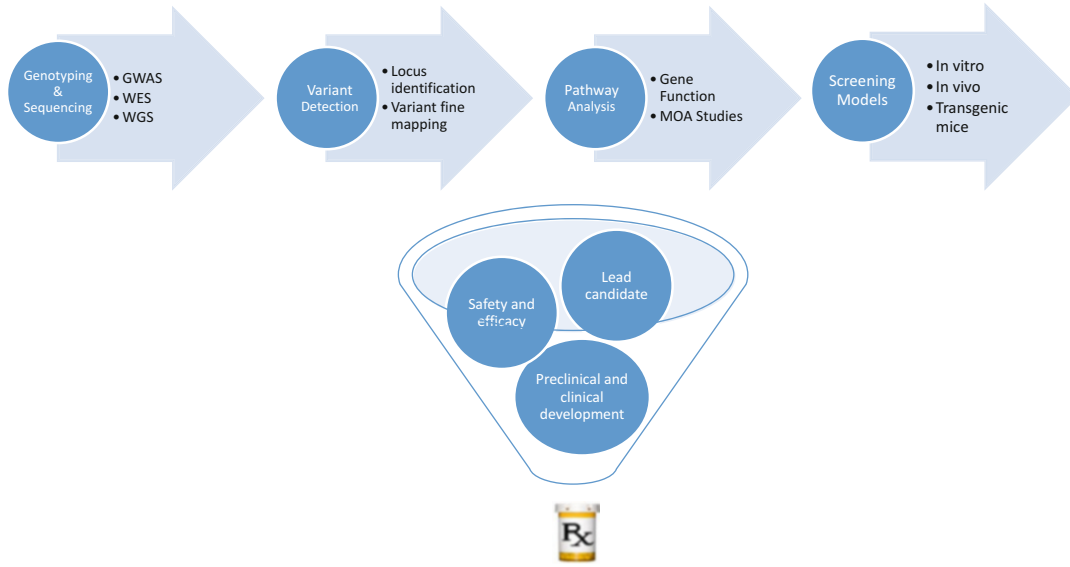

Fig. 37.3 A schematic flow diagram for drug discovery using genetic signatures. A schematic diagram representing the drug discovery using genetic signatures. *GWAS* genome-wide association studies, *WES* whole-exome sequencing, *WGS* whole-genome sequencing

well as to validate the drug target. Relevant animal models of a disease could greatly enhance the development of new therapies. AMD models have been created in mice, rats, rabbits, pigs, and nonhuman primates. Rodent models are less costly compared to nonhuman primates and offer the ability of easier genetic manipulation. However, anatomy of rodent eye differs from human eye since there is no macula in the rodents. On the other hand, nonhuman primates offer the model that is anatomically closest to human eye but are more expensive and difficult for genetic manipulations. Most commonly used animal model for AMD drug discovery is laser-induced CNV [60, 61], where high-powered, focused laser energy is used to induce a break in Bruch's membrane. However, this model cannot be used to study the role of gene mutations in developing AMD/PCV. A different approach needs to be used to investigate the role of gene mutations in AMD/PCV and validating the target for drug discovery. Therefore, the first step in the drug discovery using newly identified/validated gene as a target will be to develop an animal model carrying the genetic variations and investigate the role of the particular gene in developing AMD/PCV. For example, although there are several CETP transgenic mice models described in the literature [62–64], none have looked at the effect of D442G mutation on the development of AMD phenotypes.

In summary, genetic associations with AMD differ between Asian and Caucasian populations. Three new loci were recently found that are strongly associated with exudative AMD in Asian population, and a specific mutation Asp442Gly (rs2303790) was found in CETP, a known locus for AMD, that is strongly associated with AMD (OR 1.70) in Asian population [47]. The association of Asp442Gly (rs2303790) in AMD is the strongest outside the two most common variations found on chromosome 1 and 10. Several studies have also found CETP genetic variants associated with a high risk of PCV, thus suggesting possible involvement of the HDL metabolism in the pathogenesis of PCV and AMD in Asians [53, 54]. These findings could potentially be helpful in identifying a novel therapy for AMD/PCV treatment, specific to Asian populations.

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A Perspective: How Gene Therapeutics Can Help Address Global Health Issues

38

Paul L. Kaufman

Abstract

Current treatment for glaucoma is directed at reducing IOP, primarily via small molecules applied topically by the patient. Management of the disease is problematic for many patients but is a greater burden for those in resource poor areas. Glaucoma management often requires that the patient instill multiple topical drop medications each day, travel significant distances to frequent clinic visits and have access to a pharmacy or other storage/dispensing facility. A better approach is needed that provides a one time for a long time, or a one time for all time, administration of a therapy or medication. While there may be one ophthalmologist per 10,000 people in the United States, there may be only one per million in parts of Africa. Gene therapy has the potential to play an especially important role for underserved patient populations. Gene therapy strategies that target trabecular meshwork (TM)/Schlemm's canal (SC) cells to enhance conventional outflow, ciliary muscle cells to enhance uveoscleral outflow, ciliary body nonpigmented endothelial cells to reduce aqueous humor formation, or RGCs to impede the apoptotic cascade could all benefit glaucoma patients.

Keywords

Glaucoma • Gene therapy • Global health disparities • Prevention of blindness

Glaucoma, a characteristic family of optic neuropathies, is the first or second most common cause of irreversible vision loss globally [13]. In the United States, it ranks second overall, and first among African-Americans [17]. Prevalence models have estimated that the most common form, primary open-angle glaucoma (POAG), afflicted approximately 60.5 million people worldwide in 2010 [11], and this number is expected to increase to 79.6 million by 2020 [15].

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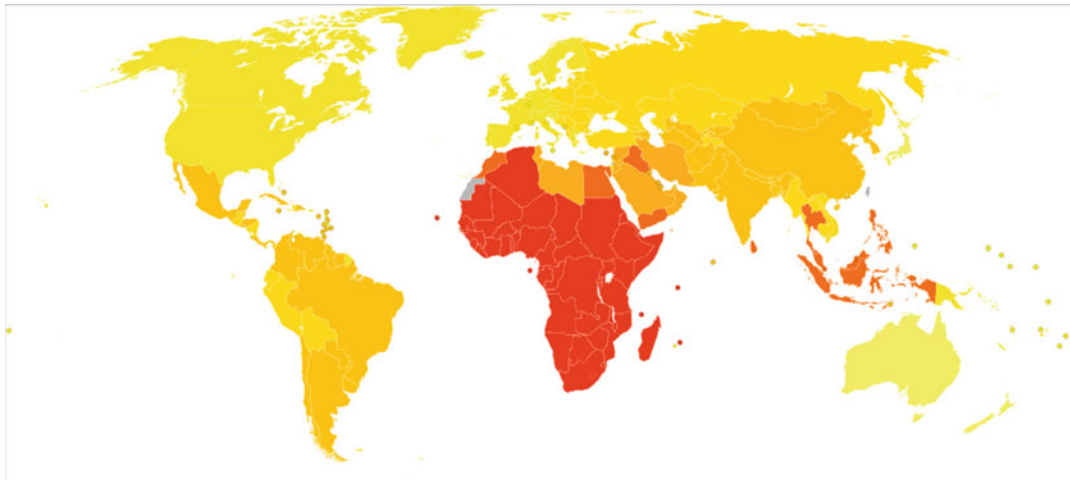

Age-standardized disability-adjusted life year (DALY) rates from glaucoma by country (per 100,000 inhabitants)

[Vector map from BlankMap-World6, compact.svg by Canuckguy et al.
Data from *Mortality and Burden of Disease estimates for WHO member states in 2002* (2009–11-07)
Combined by Lokal_Profil (author)
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The major risk factors are older age [4], black or Latino heritage [7, 10, 14], thinner central cornea ([6], larger vertical cup-to-disc (C/D) ratio), larger vertical C/D ratio asymmetry, higher visual field pattern standard deviation (PSD) [3]), strong family history [7], and intraocular pressure [4]. At age 75, white Americans have a 5% chance of having POAG; African-Americans, a mixed-race group, have a 10–12% risk; and Afro-Caribbeans, a more pure black racial group, are at a 20–25% risk [4, 7, 16]. Latino groups are at equivalent risk to African-Americans, perhaps reflecting their multiracial origins [17]. These risk factors are all non-modifiable. The only modifiable risk factor is intraocular pressure (IOP). IOP is a risk factor, a causal risk factor and a cause at every level of IOP, including IOP in the statistically normal range [8, 18]. All current treatment is directed at reducing IOP, primarily via small molecules applied topically by the patient. Often, multiple medications are needed. The disease is especially severe, early onset, and difficult to treat in black Africa, where it has devastating effects.

Treatment efficacy, monitoring, and adjustment require frequent clinic visits, and medications must be renewed frequently requiring proximity to a pharmacy or other storage/dispensing facility. In many parts of the world, access to ophthalmic care and drug dispensaries is difficult. Distances are great, roads are poor or nonexistent, drugs are not affordable, and service providers are few. While there may be one ophthalmologist per 10,000 people in the United States, there may be only one per million in parts of Africa [12]. In parts of the world where angle-closure glaucoma is the most common form, topical medical therapy is still important and is likely to remain so, even with the advent of laser treatments, improved glaucoma drainage procedures, and microinvasively implantable drainage devices and hopefully with direct neuroprotective therapies to work in concert with IOP-lowering agents.

As long as the patient remains part of the delivery system for relatively short-acting topical molecules, therapeutic access will be problematic in many parts of the world for many millions of people. This is especially true in most of Africa and in many parts of Asia and Latin America. Compliance with/adherence to complex topical medical self-administration regimens are especially problematic in poor rural households leading hardscrabble lives. Storing and administering medications under hot, dusty or humid conditions is very difficult, particularly for patients who are elderly and/or easily confused. A better approach that provides a one time for a long time, or a one time for all time, administration of a therapy or medication would be especially well suited for these situations.

Gene therapy may play an especially important role under such conditions. In this context, gene therapy means reprogramming specific cells in a patient's eye to make more or less of something so as to up- or downregulate a physiological process resulting in a reduced IOP or an inhibited retinal ganglion cell (RGC) apoptotic cascade. Targeting trabecular meshwork (TM)/Schlemm's canal (SC) cells to enhance conventional outflow, ciliary muscle cells to enhance uveoscleral outflow, ciliary body nonpigmented endothelial cells to reduce aqueous humor formation, or RGCs to impede the apoptotic cascade could all benefit glaucoma patients. Specific targets based on what we already know from small molecule clinical pharmacology could comprise (1) inducing ciliary muscle cells to make more cyclo-oxygenase-2 (COX-2) intermediary, prostaglandin $F_{2\alpha}$ (PGF) synthase, FP--prostanoid receptor mRNA, and certain post-transcription regulatory elements (all mimicking topical prostaglandin analogue therapy); (2) inducing TM cells/SC endothelial cells to make less rho kinase or myosin light-chain kinase or more myosin light-chain phosphatase (mimicking rho kinase inhibitor therapy); and (3) inducing the ciliary NPE to make less carbonic anhydrase (mimicking carbonic anhydrase inhibitor therapy), all by making more or less of a regulatory enzyme critical for these pathways. Genes or siRNAs or shRNAs may be transferred in various manners, most typically via specially engineered viral vectors. This has proven effective in glaucoma therapeutically relevant cell cultures (TM/SC) and organ cultures (TM/SC) and, at least for reporter genes, even in live nonhuman primates (NHP) [5, 9]. Reporter genes have been expressed in live NHP trabecular meshwork and ciliary muscle for over 2 years without adverse ocular or systemic effects [1, 2].

One can envision a single intracameral or intravitreal injection of vectors that will reduce IOP or inhibit retinal ganglion cell somal and axonal death for years, without frequent visits to ophthalmic centers. In the future, it is quite possible that IOP measurements and optic nerve structural and visual function tests could be obtained in local/regional health centers by modestly trained local technologists and transmitted electronically to regional or national reading centers where they could be interpreted by consultant ophthalmologists, thus eliminating difficult journeys for patients to visit faraway centers, except for major interventions. Such "telemedicine" initiatives are well underway in both the developing and the developed world, often in collaboration. Even major therapeutic medical interventions, such as "adding" another genetic

agent or "turning off" an existing one, could be undertaken by a competently trained local "injection" technician. Many of us have encountered extraordinarily intelligent, motivated, and gifted individuals in the remotest villages and the most neglected inner cities who could be trained and empowered to serve their own and surrounding communities in this manner, along with administering and transmitting the diagnostic imaging and psychophysical tests needed to follow these patients. They would be one part of a local + remote eye care network that brings high-tech medicine – diagnostic and therapeutic – to a low-tech world at rational cost to donors, local and national governance, and would facilitate not only the most modern ophthalmic glaucoma care but also community and individual self-respect to those most in need of all of those. Administration of genetic agents and constructs using tissue-selective promoters might not even require injection in the future – they might be given topically as eye drops or by corneal iontophoresis.

While there are many caveats and constraints before these visions can be realized, the objectives are plausible and feasible. Such scientific achievements might arise from research in highly developed nations, but the greatest need to apply them will be among the most underserved, financially and logistically challenged populations, mostly in Africa and large parts of Asia, where the standard therapeutic options of patient self-administered topical small molecules face many obstacles. In vitro cell and organ culture and in vivo animal studies can often provide sufficient safety and efficacy data for early-phase clinical trials, which can then be conducted in highly developed nations that have the infrastructure and are equipped to assess treatment efficacy and to monitor for expected and unexpected side effects and safety issues. According to the FDA's Considerations for the Design of Early-Phase Clinical Trials of Cellular and Gene Therapy Products: Guidance for Industry, 2015, early-phase clinical trials should provide information about general safety (including recording of symptoms and common clinical measurements, such as physical examinations, chemistry profiles, complete blood counts, and possibly other examinations that are appropriate for the condition being investigated), as well as information regarding efficacy and pharmacologic activity. The specific length of time and frequency for monitoring would depend on results of animal studies, the specific product, and/or other factors, but this could be done in nations that already have the appropriate infrastructures to do these types of trials. Once the safety and efficacy of gene therapy treatments has been established, they could be exported to regions that are most in need and later phase clinical trials done in the targeted populations. Such treatments would be determined to be safe and effective in first world nations prior to exporting them to developing countries, such that third world or developing countries would not be used as "guinea pigs" for treatments that are only available and used by first world countries. Although the science and infrastructure to realize these visions is not yet "there," one hopes, for the benefit of millions, that these days are not too far ahead of us.

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About the Editors

Dr. Gyan Prakash

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 National Eye Institute in the US since 2012, and worked across interdisciplinary and geographic boundaries to lead strategic programs and opportunities for advancing scientific research, training, developing next generation of biomedical workforce, and promoting global engagement of researchers at all career stages. Earlier in his career, he worked with the industry leaders to create three new companies (Mesa Diagnostics in Albuquerque, New Mexico, Metastatin Pharmaceuticals in Bethesda, Maryland, and AMAR International in Reston, Virginia), led teams at established companies (Pfizer International in New York, Johnson and Johnson/Ortho Diagnostics in California, and PRA International in Virginia), and mentored the next generation of global health scientists and R & D managers at NIH-Global Health Interest Group (NIH-GHIG), George Mason University, University of Maryland and Johns Hopkins University.

Dr. Prakash created and established over a dozen innovative and fully operational product development programs in the pharmaceutical and biotechnology industries. He served as the Project Scientist for Pfizer's first therapeutic biologic (first therapeutic monoclonal Antibody – E5), and then for fluconazole, the number one selling antifungal drug in the world. Dr. Prakash earned his first international program management experience working at the Pfizer world headquarters in New York in many leadership roles over several years. At Pfizer, he administered thirty-four international clinical programs across the globe for the largest selling antifungal drugs in the world, fluconazole and voriconazole, which have been used by millions of HIV/AIDS and cancer patients and saved millions of lives around the world in the immunosuppressed and immunocompromised patient populations. While at Pfizer and then at NIH-NIAID, he worked with the clinical investigators and scientists in low and middle income countries on various programs related to infectious and neglected tropical diseases such as TB, malaria, influenza and visceral leishmaniasis. His experience in non-communicable diseases spans from eye diseases, cancer, and drug and substance abuse. Before joining the National Institutes of Health, he served as the Chief Operating Officer and Senior Fellow at AMAR International, Inc., a life science program management company in Reston, Virginia, USA, where he led biomedical program management teams for the US Government for large programs. Previously, Dr. Prakash served as the President and CEO of Metastatin Pharmaceuticals, Inc., a cancer biologics-development stage company in Bethesda, Maryland, USA.

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Dr. Prakash has served in the capacity of senior scientific advisor for several public and private pharmaceutical/biotech companies, as Director for the programs of American Society for Microbiology and a member on national committee for a major medical and professional association. Dr. Prakash held an Adjunct Professorship at Robert H. Smith School of Management, University of Maryland and served on the advisory board of New Market Growth Fund of Dingman Center for Entrepreneurship. In 2001, he was appointed an Adjunct Professor at School of Management at George Mason University in Virginia where he established the first graduate program on bioscience management in the US. 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 Decker of New York. He has also coauthored a manual of business of biotechnology and has presented numerous invited lectures around the world.

Dr. Prakash earned his graduate degrees, MS and Ph.D. in Medical Microbiology (University of Illinois at Urbana-Champaign, USA) and an MBA in Pharmaceutical Management/Marketing (St. Joseph's University, USA). He received professional training at the UCLA School of Management and Centers for Disease Control (CDC) in Atlanta. Prior to immigrating to the US in 1978, Dr. Prakash earned a BS (Biology and Chemistry) and a MS (Biochemistry) at University of Allahabad in India. He received the University Gold Medal in the University for securing the highest rank in the undergraduate program. Dr. Prakash has received a number of national and international awards and recognitions in his career including a UNESCO Fellowship, AAAS award, the White House Blue Ribbon Award, Dr. Brahma Prakash Memorial Oration Award and the Aditya Jyot Foundation Award. He has served as invited speaker in many parts of the world, such as Japan, India, China, Brazil, Australia, the UK, Spain, France, Germany and many other countries. Dr. Prakash has been affiliated with several leading universities in the US 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 in elementary schools and was featured on "The Frontline" at the Public Broadcasting Service (PBS).

Dr. Takeshi Iwata

Dr. Iwata received his Ph.D. from the Department of Agriculture at Meijo University in Japan and moved to the National Eye Institute (NEI)/National Institute of Health (NIH), USA, as postdoctoral fellow in a retina genetic laboratory, headed by Dr. George Inana. The laboratory had just identified the first gene responsible for genetic eye disease called gyrate atrophy caused by a mutation in ornithine aminotransferase (OAT) gene. Dr. Iwata's first job was to use this OAT cDNA probe to hybridize OAT pseudogenes in X-chromosome and perform linkage analysis for X-linked retinitis pigmentosa families. He moved with Dr. Inana to Bascom Palmer Eye Institute, University of Miami School of Medicine in Miami, Florida to further work on other hereditary retinal diseases. After 2 years, he returned to the NEIlaboratory headed by Dr. Deborah Carper to work on another major retinal disease, the 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 work in the US motivated him to significantly expand the research when he returned to Tokyo, Japan to head the first laboratory at the National Institute of Sensory Organs (NISO), Tokyo Medical Center, National Hospital

Organization (NHO). The NHO is a group of 143 National Hospitals with 52,000 beds and more than 59,000 total employees. Ten Research Centers in Japan are affiliated to the NHO and located in major cities with each center targeting specific medical research area. The NISO was developed as the first sensory organs research institution in Japan to focus on vision, hearing and vocalization research.

Dr. Iwata is currently running three main projects related to age-related macular degeneration (AMD), normal tension glaucoma (NTG) and hereditary retinal diseases as the Director of the Molecular and Cellular Biology Division. His research goes beyond identification of disease-causing gene to explore the mutant biological behavior *in vitro* and *in vivo*. His laboratory recently identified the abnormal transcriptional regulation of *HTRA1* gene at the most highly associated genome region on chromosome 10 (Iejima et al., J Biol Chem 2014). This hypothesis was confirmed when *HTRA1* was overexpressed in mouse which lead 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 world-wide clinical trial (Minegishi, Nakayama, Iejima et al., Prog Retin Eye Res 2016). In 2011, Dr. Iwata started with six Japanese ophthalmologists from different universities specialized in retinal electrophysiology to systematically analyze the genetics of family with hereditary retinal diseases. This small group has now expanded to 26 universities and institutions as the Japan Eye Genetics Consortium (JEGC) for Hereditary Retinal Diseases. A diagnostic system and a database developed and described in Chap. 2 has inspired him to help establish the Asian Eye Genetics (AEGC, http://asianeyegenetics.org) using the same system. He now serves as the president of AEGC.

Dr. Iwata has received awards from Cooperative Cataract Research Group, National Foundation for Longevity Science, Foundation Preventing Blindness, Japanese Association for Complement Research, Japan Retinitis Pigmentosa Society, International Society for Eye Research (ISER) Special Recognition Award and has given keynote lectures around the globe including major universities in the US, UK, China, India and recently at RD 2016 meeting in Kyoto, Japan. Dr. Iwata has served as a committee member in number of local and international organizations including the Association for Research in Vision and Ophthalmology (ARVO) and the International Society for Eye Research (ISER). He has served as a committee member of the ARVO Foundation Award Committee, ARVO Global Research Training Committee and ARVO Executive Committee Member for the Advocacy Pillar. He has served two terms as a counselor for ISER and recently served as the vice president (Asia & Pacific) and program chair for the XXII Biennial Meeting of ISER 2016 in Tokyo, Japan. Dr. Iwata has over 100 publications in scientific journals, reviews and book chapters. He currently serves on the editorial board for Journal of Ocular Biology, Diseases and Informatics and Eye and Brain.

Dr. Iwata has supported international collaborations between researchers, laboratories, institutions and consortia. Recently, he played a major role in establishing several Collaborative Research Agreements including the ones between NISO and NEI in the US, Buffalo Niagara Medical Center Campus in the US, Aditya Jyot Eye Hospital, Mumbai in India and Moorfield Eye Hospital-University College in London in the UK. These collaborations have brought fruitful results to NISO-Tokyo Medical Center. . As the president of AEGC, Dr. Iwata is actively involved in identifying the key leaders in the Asian region to build effective plans for future genetic eye research in Asia. He led the third annual meeting of AEGC during ARVO

2016 in Seattle with over 45 attendants representing each region of Asia. The meeting participants agreed to share eye genetic information by constructing a common AEGC database containing useful genotype-phenotype information along with natural history of patients for each mutation recorded. AEGC database for Stargardt's Disease and Occult Macular Dystrophy started operation in September, 2016 initiated by Dr. Kaoru Fujinami. These AEGC database should accelerate therapeutic developers to decide which gene to focus and who to contact for patient access. Dr. Iwata has worked with Dr. Gyan Prakash since 2013 to build eye genetic research programs in Asia. This book is not only a landmark achievement for AEGC, but it serves as a guiding document for future research programs for AEGC.

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