A challenge to the striking genotypic heterogeneity of retinitis pigmentosa: a better understanding of the pathophysiology using the newest genetic strategies

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Abstract

Retinitis pigmentosa (RP) is a group of inherited retinal disorders characterized by a complex association between tremendous genotypic multiplicity and great phenotypic heterogeneity. The severity of the clinical manifestation depends on penetrance and expressivity of the disease-gene. Also, various interactions between gene expression and environmental factors have been hypothesized. More than 250 genes with ~ 4500 causative mutations have been reported to be involved in different RP-related mechanisms. Nowadays, not more than the 50% of RPs are attributable to identified genes, whereas the rest of molecular defects are still undetectable, especially in populations where few genetic screenings have been performed. Therefore, new genetic strategies can be a remarkably useful tool to aid clinical diagnosis, potentially modifying treatment options, and family counseling. Genome-wide analytical techniques (array comparative genomic hybridization and single-nucleotide polymorphism genotyping) and DNA sequencing strategies (arrayed primer extension, Sanger sequencing, and ultra high-throughput sequencing) are successfully used to early make molecular diagnosis detecting single or multiple mutations in the huge heterogeneity of RPs. To date, further research needs to be carried out to better investigate the genotype/phenotype correlation, putting together genetic and clinical findings to provide detailed information concerning the risk of RP development and novel effective treatments.
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Introduction

Retinitis pigmentosa (RP) embraces a heterogeneous group of retinal disorders characterized by progressive retinal degeneration due to alterations of photo-receptors (PRs) and retinal pigment epithelium. The most frequent symptoms are night-blindness and the growing impairment of visual field, perceived as tunnel vision. The increasing damage of the rods determines the severity of these symptoms. Also, severe alterations of the cones cause variable visual impairments. Clinical hallmarks consist of bone-spicule deposits, waxy optic disc, and shrinked retinal vessels.

In the different ethnic groups, the prevalence of RP is variable and reported in 1 case for each 3500–5000 individuals.[1–6] On the basis of the clinical presentation, there are two main groups: typical RP or rod-cone dystrophy (~80–90%), in which the most injured PRs are the rods, and atypical RP or cone-rod dystrophy (~10–20%), in which the cones are the primarily damaged PRs.[7]

In the most part of affected individuals (~85% of cases), the inherited retinal dystrophies are isolated disorders and it is referred as non syndromic RP. However, in some patients (~15% of cases) PRs disorders are the epiphenomenon of multi-systemic manifestation of a specific syndrome (ie, Usher syndrome, Bardet-Biedl syndrome, Laurence-Moon disease, Alstrom disease, Kearns Sayre syndrome, Cockayane syndrome, Refsum disease, Bassen-Kornzwig syndrome, Friedreck ataxia, Mucopolysaccharidosis) and it is referred as syndromic RP.[8]

Clinical manifestations

In patients affected by early-stage typical RP, a decreasing of the visual abilities in the dark (nyctalopia), a slight to moderate shrinking of the visual field, and/or a remarkable dazzle sensation (photophobia) are often reported. This latter symptom is commonly detectable also in patients suffering from atypical RP. Moreover, because the cones are prevalently injured, these individuals usually complain about noticeable alterations of both quantity and quality of their central vision. Although many patients with late-stage typical RP have peripheral or total blindness and numerous patients with late-stage atypical RP are centrally blind, the natural history of all tapeto-retinal degenerations is often unpredictable.[8,9] In fact, the final visual prognosis of each RP patient can be dependent not only on genetic factors (different expressivity and/or penetrance of the causative disease-gene) but, sometimes, also on environmental factors (different levels of eye-exposure to harmful light radiations).[10–13]

Clinical signs found at ophthalmic fundus examination are characteristic: 'bone-spicule' pigment deposits in the mid periphery along with retinal pigment epithelium (RPE) dystrophy and/or atrophy, attenuation of retinal vessels, variable pallor of the optic disc, and relatively spared macula surrounded by a peri-macular ring of depigmentation.[14] In several young adults with RP, posterior subcapsular cataract, cystoid macular pattern with variable vitreo-retinal tractional component, and intra-retinal deep white dots secondary to RPE degeneration are manifest. The age of onset, the rate of progression, and the severity of the disease are extremely variable depending not only on the genetic background but also on some still unknown influencing factors.[15] Symptoms may start in childhood as well as in adulthood. Even though the progression of the disease is unpredictable, severe visual impairment typically occurs by the age of ~ 40–50 years. This large phenotypic variability can be partially explained by the huge genetic heterogeneity.[16]

Genotype–phenotype heterogeneity

There is a complex association between genotypic multiplicity and phenotypic heterogeneity. The severity of clinical manifestations is related to both penetrance and expressivity of the disease-gene. However, numerous interactions between gene expression and environmental factors have also been assumed. Even though ~ 4500 mutations in more than 250 genes are hitherto described in patients with inherited retinal dystrophies, the molecular defect is detectable in a variable percentage of cases.[17,18] Combining results from conventional Sanger sequencing and targeted-capture next-generation

sequencing (NGS), using rough estimates, it is possible to detect mutations in 20–30% of autosomal recessive forms, 60–70% of autosomal-dominant forms, and 80–85% of X-linked forms.[19] Especially in ethnic groups originated from a genetically unexplored geographic area, the percentage of identification of known disease-causing mutations should be significantly lower.[20] Moreover, individuals with the same mutation might be affected by different clinical manifestations.[21] Different RP gene-mutations trigger a huge number of alterations in such delicate molecular mechanisms: phototransduction cascade, vitamin A metabolism, structural or cyto-skeletal functions, interactive cell-cell signaling or synaptic interaction, intron-splicing of RNA, intracellular trafficking, maintenance of cilia/ciliated cells, and PR disks engulfment.[22,23] In fact, Daiger and co-workers claim that the extent of heterogeneity of RP can be confusing to patients and clinicians alike, and it is a confounding aspect for diagnosis. Four different types of heterogeneity can be highlighted: genetic, allelic, phenotypic, and clinical. The first occurs when different genes might provoke the same disease phenotype. The second implies that there can be many various disease-causing mutations in each gene. The third means that distinct mutations in the same gene are able to lead to different diseases. The fourth represents the fact that the same mutation in different subjects can result in various clinical consequences, even in people from the same family.[17]

The genetics of RP is definitely multifaceted. The inheritance can be autosomal dominant (20–25%), autosomal recessive (15–20%), or X-linked (10–15%). Some RP forms can be sporadic (30%) or early onset (5%) and classified as part of Leber congenital amaurosis. Moreover, there is quite a small group of RPs which are very rare such as mitochondrial and digenic forms.[24,25]

Ophthalmic diagnostic exams

The mainly used exam for the screening of patients with RP is the visual field (VF) testing mapping central and peripheral vision. VF patterns are quite characteristic. In early stages a patchy loss is typically located in mid-periphery 20–25° from fixation and, changing over time, it evolves into a ring scotoma that enlarges mostly toward the periphery than centrally.[14] However, the conventional perimetry is not the gold standard testing to finely evaluate the function of the macular area of RP patients who normally have unstable or extrafoveal fixation. In fact, the more performing test should be the microperimetry, which allows to assess the visual sensitivity at any specific point on the retina with accurate test–retest reliability for the same point. To establish the

location and the stability of fixation is relevant for the evaluation of the disease progression.[26] The microperimetry has been revealed innovative in comparing retinal morphology with visual function, so it can be successfully used to follow-up. Indeed, some investigators have found a significant correlation between the length of the inner/outer segment line at spectral domain optical coherence tomography (SD-OCT) and the central retinal sensitivity.[27] In patients with RP, border-line sensitivity can be observed in parafoveal regions, whereas progressively decreased sensitivity is detected moving off peripherally.[28]

Another exam of paramount importance is electroretinography (ERG). Full-field ERG measures retinal electrical potential after light stimulation. A dim blue light single flash evokes the rod response, whereas a flickering white light induces the cone response. Conventional ERG demonstrates the entire functional status of PRs detecting abnormalities just in the case of more than 20% of the retina is affected. Multifocal ERG is also more selective in localizing limited areas of retinal dysfunction and, generally, shows a significant drop in paracentral retinal function.[29]

Surely, SD-OCT is of notable importance, allowing the morphological evaluation of the inner and outer retinal layers. Some authors have found a sort of correspondence between the decreasing of the VF sensitivity and the flattening of the PR layer.[30,31] The SD-OCT allows for the intra-retinal microstructures and pathological changes in the retinal framework. During the progression of RP, the integrity of PRs is impaired and the hyperreflective IS/OS line appears to be interrupted or damaged driving away from the fovea.[32] Furthermore, SD-OCT screens patients seeking for subclinical cystoid macular edema which has a prognostic value on the central visual acuity.

Fundus autofluorescence (FAF) is a non-invasive imaging technique that offers another approach for analyzing the macular region. In retinal dystrophies as well as in RP, arcs and rings of hyperautofluorescence have been described around the central retinal area.[33] The increased autofluorescence is associated with disruption of the IS/OS junction and thinning of the outer retinal layer on SD-OCT.[34] The pattern of FAF results from an abnormal accumulation of both lipofuscin in the RPE due to a progressive outer segment dysgenesis and other fluorophores in the PR layer, such as an excessive production of bisretinoid following an abnormal handling of vitamin A aldehyde.[35] The inner border of the hyperautofluorescent ring spatially corresponds to the lateral extent of the undamaged IS/OS junction. Inside this ring the retinal sensitivity is rather preserved, whereas outside it is markedly decreased.[36]

Molecular pathways for PRs cell death

RPE and choroid provide metabolic support to the PR cells. Therefore, when physically detached from the underlying RPE, the PRs go toward death. Owing to the degeneration of RPE cells or abnormalities of choroidal vessels, PRs progressively die.[37] Causes and clinical findings differ one disease from another, but the underlying biochemical mechanisms involved in the PR degeneration largely share common molecular pathways. On the basis of the morphological appearance at ultrastructural studies, cell death has been classified in three main forms: apoptosis, autophagy, and necrosis.[38] The apoptosis is morphologically characterized from condensation of nucleus and cytoplasm, rounding-up of the cell, reduction of cellular volume, and engulfment by resident phagocyte.[39] Biochemical evidence has highlighted not only the role of caspase-dependent pathways but also of caspase-independent pathways (apoptosis-inducing factor (AIF), endonuclease G, cathepsins, calpains, polyADPribose polymerases (PARP)).[40] Autophagy is the process by which cellular macromolecules (proteins, lipids, and nucleic acids) and organelles (mitochondria) are digested by the lysosomes.[41] Transmission electron microscopy can appreciate the formation of large inclusions (autophagosomes and autolysosomes) in the cytoplasm with lack of condensation and fragmentation of cells.[39] Necrosis is a biochemical process mediated by the activation of receptor-interacting protein 1 and 3 (RIP1 and RIP3). Recent studies revealed the importance of the 'RIP kinase-dependent necrosis' in retinal degeneration.[42] Ultrastructural studies in necrosis showed a swelling of cytoplasm and organelles, a gain in cell volume, and plasma membrane rupture with connections with the extracellular cavity.[39]

In different models of RP, naturally occurred or genetically manipulated, Chang et al.[43] demonstrated that rods might undergo apoptosis when there are mutations in phosphodiesterase 6beta, peripherin, and rhodopsin (RHO). Other studies investigated the role of caspase activation for the PR death. Caspases are initially produced as catalytically inactive zymogens and then they are activated by proteolytic cleavage or allosteric conformational changes. The excessive exposure to light induces apoptosis in PRs because of overactivation of phototransduction pathway and accumulation of 11-cis retinal, the chromophore of rod and cone opsins. Also, there is a caspase-independent pathway inducing apoptosis by activation of AIF, a flavoprotein located in the mitochondrial inter-membrane space and involved in the respiratory chain and oxidative phosphorylation.[44,45] In models of RP, nuclear translocation of AIF following an

increased activity of calpain and PARP has been observed in dying PRs.[46,47]

Genetics

Recent fine-detailed reviews on RP gene mutations have been published.[16,17] The molecular genetics of RP is definitely very complex. To date, research is investigating an increasingly huge number of new identified gene mutations, either sporadic or familiar. RP-associated genes code for proteins involved in phototransduction, visual cycle cascade, PR transcription, and structure.[20] Nevertheless, different mutations in the same gene can lead to different phenotypes and the same mutation just in one gene can also elicit variable diseases. For instance, the most of RHO mutations show an autosomal-dominant inheritance while a few RHO mutations show an autosomal recessive inheritance.[48] Likewise, RP GTPase regulator mutations have been found in males since it is mainly involved in X-linked recessive RP, but also female carriers with symptoms are detected in families with X-linked dominant RP.[49] Moreover, in monogenic RP some genes are characterized by incomplete penetrance and by different modes of transmission.

Early onset RP, diagnosed around 2 years of age, is mainly characterized by mutations in the causative genes RPE65, CRB1, and TULP1.[50–52] Late onset RP, diagnosed in adulthood and typically characterized by autosomal-dominant inheritance, is mostly correlated with the involvement of the CRX gene.[53] Subjects affected by myopia, with retinal pigment atrophy, or by RP sine pigmento usually present little of peripheral pigment deposits and this finding has no prognostic value.[15] On the other hand, gene variants are associated with specific fundus findings as following. Mutations in the RHO and PRPF31 genes are typical for sector RP.[54] The CRB1 gene correlates to para-arteriolar retinal pigment epithelium preservation, whereas RLBP1 gene mutations lead to retinitis punctata albescens.[55,56] RDS and CRX genetic alterations generally result in severe macular atrophy.[57,58]

Recent genetic strategies for molecular diagnosis

The human genome is characterized by two main types of variation: single-nucleotide variation, which is DNA base-pair substitutions and short indels, and structural variation, which means alteration of many DNA base pairs (ie, inversions, translocations, insertions, deletions, and duplications) resulting in copy number variation (CNV).[59] The array comparative genomic hybridization (array CGH) and the single-nucleotide polymorphism (SNP) genotyping are modern genome-wide analytical techniques. Over the last years, such genetic procedures have been very useful to detect an increasingly huge number of CNVs and, often, they have been utilized by clinicians for the assessment of patients with genetic disorders, such as inherited retinal distrophies.[60,61] Genomic resolution by both array platforms (SNP and array CGH) permits the detection of genomic gains and losses of ~ 400 kb in size.[62]

Custom-designed oligonucleotide array CGH allows for the detection of single-exon CNVs for clinically relevant genes.[63] This genetic technique expands the diagnostic yield, yet it raises the probability of detecting variants of debatable clinical significance. SNP arrays show high sensitivity for the detection of low-level mosaic aneuploidies and chimerism and allow to detect copy number neutral regions of absence of heterozygosity (AOH).[64] AOH might reveal the consanguinity because multiple segments of AOH, making up regions on a matching pair of chromosomes, are identical by descent in inbred populations. The size and number of AOH blocks correlate with the degree of parental lineage. Therefore, in the clinical practice the localization of homozygous regions is used to detect autosomal recessive disease-causing genes. In ethnic populations, the inbreeding is quite common, so that numerous recessive RP mutations have been identified by means of homozygosity mapping.[65]

Nowadays, there are several DNA sequencing techniques successfully used for molecular diagnostics of various genetic disorders and fine-tuned to detect single or multiple mutations, such as arrayed primer extension (APEX), Sanger sequencing, and ultra high-throughput sequencing.[17] APEX is a microarray-based genotyping technique that permits to simultaneously analyze hundreds of known mutations in the genome.[66,67] DNA sequencing is a method that determines the order of DNA nucleotides and it is used to test for genetic disorders. The Sanger sequencing has been automated and made it faster over the last years. At present, it is still used in laboratories to sequence short pieces of DNA, but it remains rather expensive and time-consuming, and it cannot be used to sequence the human genome. The newest developed technology is the NGS that strongly speeds up the process of cutting down the costs.[68,69] It 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.[70] This method allows for fast analysis of large portions of the human genome, but it is not applicable to some features of DNA (deletions, rearrangements, expanded repeats, and haplotypes). The NGS technique enables to analyze exons, which are DNA pieces that carry instructions to build proteins making up ~ 1.5% of the entire human genome. The set of all exons in a genome is called exome. However, DNA variations outside the exons have been

thought to affect gene activity and protein production leading to genetic disorders.[71] There are three main NGS strategies: whole-exome NGS, whole-genome NGS, and targeted-capture NGS. The first-sequencing technique relies on the capture of all protein-coding regions, that is all exons. Thus, by definition this method is able to detect mutations only in coding regions of any gene to be identified, but it cannot select few genes. As most known RP mutations occur in exons, whole-exome NGS might be considered an efficient method to identify possible disease-causing mutations. The second technique scans the whole genome without distinction between exonic and nonexonic regions so that it allows the detection of intergenic variants, CNVs, and other structural DNA rearrangements.[72] It covers nearly the entire human genome (~98%) but it deals with several limitations, such as high costs, the requirement of high-quality DNA, and extremely complex analysis of the sheer volume of data acquired. The third strategy tests only the exons of still known disease-causing genes.[73] The drawback is the fact that no new genes can be discovered, but the big advantage is the opportunity of finely scanning a specific gene, also analyzing the related mutations with low costs.[74] To date, most reports describe monogenic conditions. The whole-genome NGS is a powerful tool for detecting mutations in a highly genetically heterogeneous Mendelian disease such as the RP.[75] The chance to analyze noncoding regions allows to identify some pathogenetic mutations still undetected and some complex disease-causing variants. Actual drawbacks of this genotyping sequencing technique include the requirement of high-quality DNA and the significantly big costs. Considering the fact that the majority of patients referred for molecular genetics diagnosis come from a specific ethnic group, the whole-genome NGS can be useful to identify unambiguous causative mutations among the overwhelming number of DNA variants in the human genome.[72]

Conclusions

The genetics of RP is strikingly challenging. At present, a correlation between genotype and phenotype is no longer as obscure as it was in past decades, even though still big efforts are ongoing to establish a specific correlation between genotypic heterogeneity and phenotypic variability. Genetic mutations in RP individuals are population specific.[76,77] However, in spite of the higher and higher number of identified disease genes, nearly 40–50% of RP patients present no mutation in recognized loci.[22] Also, the significance of novel huge genetic information is still often unknown. Not every genetic variation affects health, so it is hard to say whether identified mutations are actually involved in the

pathologic condition under investigation. Indeed, sometimes, a detected variant is associated with a genetic disorder not yet clinically assessed.

To sum up, new high tech genetic strategies are valuable methods for researchers and, certainly, they will be a remarkable instrument for aiding in diagnosis of genetically determined disease in the next future. Furthermore, these new genetic techniques are a striking tool to better understand the molecular basis of genetic disorders and to achieve the target of individualized medicine and tailored treatment.

Disclaimer

The authors alone are responsible for the content and writing of the paper.

Conflict of interest

The authors declare no conflict of interest.

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