Update on the Corneal Dystrophies-Genetic Testing and Therapy

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Update On The Corneal Dystrophies-Genetic Testing and Therapy

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Abstract

One major purpose of the IC3D Corneal Dystrophy Nomenclature Revision was to include genetic information with a goal of facilitating investigation into the pathogenesis, treatment, and perhaps even prevention of the corneal dystrophies an ambitious goal. Over a decade has passed since the first publication of the IC3D corneal dystrophy nomenclature revision. Gene therapy is available for an early-onset form of inherited retinal degeneration called Leber's congenital amaurosis, but not yet for corneal degenerations. We review the current state of affairs regarding our original ambitious goal. We discuss genetic testing, gene therapy (RNA interference: RNAi; genome editing), and ocular delivery of corneal gene therapy for the corneal dystrophies. Why have gene therapy techniques not yet been introduced for the corneal dystrophies?

Introduction

In the 19th century, Bücklers presented the first classification system of three forms of corneal dystrophy. In the early 20th century, introduction of the slit lamp biomicroscope allowed for more detailed examination leading to the identification and classification of distinct corneal dystrophies based on their unique phenotype and histopathology. By the late 20th and early 21st century, the advent of genetic linkage analysis allowed for the identification of the associated gene loci, and eventual localization of causative genes and pathogenic variants. International Committee of Corneal Dystrophy Nomenclature Revisions in 2008 and 2015 for the first time, included genetic information within the corneal dystrophy classification system.[1,2] The goal was "to provide(s) a better understanding of the mechanism of the disorder…and present some therapeutic possibilities." In the 21st century, we had advanced beyond phenotype and histopathologic description to also include genotypic information.

At the same time, surgical therapeutic options for opacified corneas expanded from the replacement of the entire cornea thickness to excision and replacement of the involved layer. In addition to penetrating keratoplasty, focused replacement includes deep anterior lamellar keratoplasty (DALK) for deeper stromal dystrophies and Descemet stripping automated endothelial keratoplasty (DSAEK) or Descemet membrane endothelial keratoplasty (DMEK) for endothelial dystrophies (Figure 1). Over a decade ago, at the May 2009 ARVO/Pfizer Institute on Corneal Dystrophies: Molecular Genetics to Therapeutic Intervention,[3] we discussed that the IC3D classification "was the first step" to "understanding the genetic basis of each disease". The goal to discover corneal dystrophy pathogenesis would "allow the development of nonsurgical therapeutic interventions to prevent visual loss," including gene therapy. Our retina colleagues have expanded the treatment possibilities for inherited retinal dystrophies to include gene therapy, but this has yet to occur for corneal dystrophies. In the field of hereditary corneal diseases, we may have become a victim of our success. The surgical interventions for corneal dystrophies have proven to be so successful, it is easier to wait

for vision loss with subsequent surgical intervention than to take risks associated with any new therapy, such as gene therapy.[4]

Genetic disease burdens both affected individuals and their close relatives, including their children and their children's children. When examining families with Schnyder corneal dystrophy, one of the authors (JSW) was always struck by the relative hopelessness of the early diagnosis of Schnyder corneal dystrophy in a child (Figure 2). Parents could only be told the course of visual loss with age rather than potential early interventions to prevent the visual loss. Beyond treating affected individuals, "Research is critically important to those who have the corneal dystrophies…because it offers the hope….of changing the future of innocent offspring by developing improved treatments, or even prevention".[3]

We review the current state of genetic testing and gene therapy in corneal diseases.

Differentiating the hereditary corneal dystrophy from systemic diseases with corneal involvement

Hereditary corneal disease can be an isolated phenomenon confined to the cornea or be associated with abnormalities in other parts of the eye or the body. Consequently, the evaluation of suspected genetic corneal disease must include careful examination of the entire eye. In addition, because corneal phenotypes can be part of systemic diseases, it is important to determine if there are other symptoms or signs of systemic disorders.

The ophthalmologist can give important corneal hints with regard to diagnosis of a systemic disorder in very young patients: cornea verticillata in Fabry`s disease; peripheral brownish corneal band in Wilson`s disease, punctiform corneal crystals in cystinosis; haze and peripheral ring in lecithin cholesterol acyltransferase (LCAT) deficiency; diffuse corneal haze in the different forms of mucopolysaccharidosis; pseudo-dendriform lesions in tyrosinemia II and many others.[5]

It is important to diagnose systemic disorders promptly, as the introduction of systemic enzymatic therapies for mucopolysaccharidosis has broadened the therapeutic armamentarium for the current

standard of care. Furthermore, in tyrosinemia II a lifelong dietary restriction of tyrosine and phenylalanine amino acids is necessary immediately. One example of a systemic disease with ocular findings resembling a corneal dystrophy is familial amyloidosis, Finnish type (also known as Meretoja syndrome or hereditary gelsolin amyloidosis), caused by the pathogenic variants in the gelsolin gene.[6,7] These individuals exhibit a lax, mask-like facies due to neuropathy of the facial nerve. The corneas demonstrate lattice like opacities resembling classic lattice corneal dystrophy (LCD1), which in an advanced state leads to corneal epithelial erosions and neurotrophic keratitis. However, unlike the lattice lines in LCD1, the lattice lines in familial amyloidosis are less numerous, start peripherally and spread centrally (Figure 3). Familial amyloidosis, Finnish type is important to recognize because the penetrating keratoplasty has a high risk of failure, and the patient should be well informed when considering surgery.[8]

Another example of systemic disease associated with corneal changes is monoclonal gammopathy of undetermined significance (MGUS) which can cause paraproteinemic keratopathy.[9] The corneal involvement is typically bilateral with heterogeneous appearance, from lattice lines (Figure 4) to different patterns of stromal opacities. These corneal findings can lead to misdiagnosis of corneal dystrophy.[9] However, unlike most corneal dystrophies, there is absence of both family history and onset at a young age. The diagnosis of MGUS is confirmed by serum protein electrophoresis.

Other genetic eye diseases with corneal involvement and distinct phenotypes

The genetic disorders affecting the development of the eye can produce corneal anomalies. Aniridia has a very diverse phenotypic variability from a total absence of iris to small anomalies in the anterior chamber angle leading to juvenile glaucoma. Individuals with a pathogenic variant in the *PAX6* gene have often very severe corneal abnormalities.[10] Axenfeld—Rieger syndrome, Peters anomaly, Marfan syndrome, microphthalmia, and megalocornea exhibit characteristic corneal phenotypes that should be recognized.

Corneal dystrophies

Patient examination and genetic testing

In order to determine if a patient has a true corneal dystrophy, it is essential to perform a detailed ocular checkup including examination the anterior chamber angle, lens, and posterior parts of the eye. Comprehensive imaging with photography, red-reflex photos, anterior optical coherence tomography, specular microscopy, and *in vivo* corneal confocal microscopy can lead to the correct diagnosis. Moreover, the systemic signs and symptoms should be noted.

While the family history and corneal examination are usually the mainstay of diagnosis, genetic testing provides definitive confirmation. This requires sample collection from blood or buccal swab, and subsequent DNA extraction. Prior to performing genetic testing, it is very helpful to record the family history and to know both the prevalence and penetrance of the disease in the population in relation of the existing disease-causing variants. Genetic counselling should be provided as the dystrophy could affect other family members of the patient. Although the diagnosis of a cornea dystrophy is often made purely on family history and clinical examination, it is important to consider genetic testing because only this provides a precise diagnosis, inheritance pattern, guides the treatment choice, and in the future may offer more sophisticated intervention.

If the clinical diagnosis is relatively certain, even one variant or gene could be sequenced; this is often the case with *TGFBI*-related dystrophies. Currently, the easiest way to perform genetic testing is to order the gene panel that includes the genes linked to corneal dystrophies. These panels are technically performed with exome sequencing (ES, i.e., sequencing nearly all coding regions of the genes), but only genes related to corneal disorders are analyzed, thus coincidental findings are avoided (such as cancer-predisposing variants). ES is not a reliable method to recognize larger copy-number variations, thus molecular karyotyping is sometimes needed. Posterior amorphous corneal dystrophy is caused by a heterozygous deletion of chromosome 12q21.33 covering four small

leucine-rich proteoglycan genes.[11] If the panel remains negative, ES can be performed including other family members to find new causative genes. The ES does not cover regulatory and intronic regions; therefore, whole-genome sequencing could be an alternative approach but is not yet standard clinical genetic testing.

The success rate of the gene panel testing in corneal dystrophies is relatively high, with 71% of positive results according to some authors,[12] reflecting the accuracy of clinical diagnosis and knowledge of the genetic background of these diseases.

Corneal Gene Therapy in the Corneal Dystrophies

The anatomical position and structure of the cornea make it an attractive target for gene therapy approaches[13,14]. The cornea is easily accessible and allows observation of the phenotypic effects of gene therapy approaches *in vivo* through high resolution imaging due to its optical properties and avascularity [14,15]. The cornea also shows immune privilege, which facilitates gene delivery as a therapeutic option. Gene therapy for corneal dystrophies offers benefit in the clinical management of affected individuals. The hope for gene therapy in the future is that it could be offered to younger individuals with good visual acuity and early signs of corneal dystrophy to prevent progression and visual loss. Furthermore, although surgical management to rehabilitate vision is mainstream, there is a worldwide shortage of donor corneas, all surgeries have associated risks and the underlying genetic disease can recur following surgical interventions. To develop gene-based therapies for corneal dystrophies, scientists have hijacked physiological molecular biology processes to develop therapeutic manipulations. The foundation of gene therapy is the central dogma of molecular biology in which biological or genetic information moves from DNA in the nucleus of the cell, to messenger RNA (mRNA), which is translated into protein in the cell cytoplasm. Knowledge of the pathobiological mechanism underlying the identified genetic cause of the disease is critical to establish the basis of corneal gene therapy. These methodologies must be specifically targeted to a gene and/or mutation, which means that personalized treatments will be ultimately necessary for each patient. In some

cases, concrete causative mutations have been reported with common ancestral origins or high prevalence, such as Leu132Pro and Arg135Thr in the keratin 12 gene (*KRT12)* for Meesmann epithelial corneal dystrophy (MECD), or CTG18.1 in *TCF4* for Fuchs endothelial corneal dystrophy (FECD).[16,17] These relatively frequent pathogenic variations have been the focus of several studies to develop effective gene therapy strategies, although most of these studies are still restricted to *in vitro* and *ex vivo* approaches, or *in vivo* assays in animal models.

RNA interference (RNAi) gene therapy in corneal dystrophies.

RNA interference (RNAi) can be achieved with small interfering RNA (siRNA) or antisense oligonucleotides (ASOs). The siRNA is designed to specifically target the mutant allele or mutation in an allele-specific manner (ASP-RNAi) (Figure 5). ASP-RNAi have been used in MECD[16] and the epithelial–stromal *TGFBI* dystrophies.[18] ASOs have been developed to treat the genetic mechanism underlying Fuchs endothelial corneal dystrophy (FECD), associated with a CTG trinucleotide repeat expansion (CTG18.1) in the noncoding region of the *TCF4* gene [17]. ASOs have been used *in vitro* as a functional rescue of the molecular changes in FECD and could be introduced early in the disease process to prevent progression to corneal surgery.[17]

However, RNAi approaches present some limitations, as ASP-RNAi assays are restrictively designed in cases with dominant negative mutations, in which the expression of the non-mutated allele is sufficient to recover the correct function of the gene, whereas it will be inadequate in recessive disorders or dominant cases showing haploinsufficiency. Other limitations of these strategies are the potential off-target alterations, as it is difficult to fully understand the true endogenous function of the molecule *in vivo*, and the low efficacy of the system, partly due to its incomplete and transient inhibitory effect. This variable and partial silencing knockdown is in part a consequence of the RNAi molecule sensitivity to nuclease degradation, which might involve a short half-life and high dose or a frequently repeated treatment regimen. In this regard, molecule stabilization with chemical

modifications is suggested to enhance intracellular availability and silencing persistence. Finally, effective and appropriate delivery system into corneal cells *in vivo* need to be designed.

Genome editing in corneal dystrophies.

Genome editing technologies, developed from bacterial molecular biology processes functioning as an immune system to deal with foreign genetic sequences, are being employed to develop gene therapy solutions in ocular and non-ocular genetic disorders.[13] The identification of the CRISPR-Cas9 genome editing technique resulted in the Nobel Prize in Chemistry in 2020, being awarded to Emmanuelle Charpentier and Jennifer Doudna. Cas9 (CRISPR-associated protein 9) is a RNA guided endonuclease enzyme that uses CRISPR (clustered regularly interspersed palindromic repeats) sequences as a guide to identify and cut specific strands of DNA that are complementary to the CRISPR sequence (Figure 6).[13] Allele-specific genome-editing of the Leu132Pro *KRT12* mutation was demonstrated *in vitro* and in a humanized MECD mouse model using CRISPR-Cas9 delivered by intra-stromal injection.[19] The editing efficiency was 38.5% and increasing targeting efficiency is required to further develop this approach.[19] CRISPR/Cas9-induced homology-directed repair has been employed in primary corneal keratocytes derived from a patient with granular corneal dystrophy type 2 (GCD2) resulting from a Arg124His mutation in *TGFBI.*[20] The efficiency of *in vitro* genome editing was approximately 60% for the *TGFBI* Arg124His mutant allele.[20] The best characterized CRISPR/Cas9 genome editing system is derived from *Streptococcus pyogenes* (SpCas9) and less than 30% of over 60 mutations in *TGFBI* can be targeted by this type of genome editing.[21] Using natural genetic variation in the CRISPR-Cas9 target regions on the same DNA strand as the *TGFBI* mutation supports the development of mutation independent genome.[22] Genome editing may also have a therapeutic role in FECD.[23] CRISPR/Cas9 system has some important limitations that need to be overcome before its transition towards the clinic as a therapeutic alternative, since, unlike the transient effect of RNAi methodologies, programmable nucleases introduce permanent changes in the genome.[13] In this sense, one of the major concerns is off-target effects, which refer to binding or cleavage by Cas9 at a

site other than the target site. These alterations, which have been observed at a high frequency, can disrupt the function of unexpected genes and may result in genome instability.[24] Thus, researchers need to ensure no unwanted effect is being induced, by engineering Cas9 variants and optimizing guide designs, as precise genome editing is essential for CRISPR gene therapy in patients.[25]

Ocular Delivery of Corneal Gene Therapy

Corneal gene therapy has been mainly studied in animal models, whereas the clinical trials in humans are still limited.[26] A key barrier to clinical translation is that the delivery of the genetic material must be practicable and safe, and the modulation of the corneal pathology must be durable.[27] In order to achieve optimal gene therapy efficiency, a successful delivery of the therapeutic nucleic acid into the target cell is critical. An ideal gene therapy delivery system is one that could be easily produced and provides high levels of delivered molecules in a tissue-selective manner without toxicity, immunological response, or damage. Several delivery systems, which are grouped into viral and non-viral vectors, have been tested *in vitro, ex vivo* and *in vivo* in the cornea, presenting different advantages and limitations.[26] Viral vectors are used replacing the viral genes with the nucleic acids of interest to obtain replication-defective viruses. However, most of them, including adenovirus, lentivirus and retrovirus, present important limitations concerning safety and immunogenicity. Moreover, adenovirus and retrovirus are of limited use for corneal gene therapy because of their inability to transduce low/non-dividing cells such as corneal endothelium and keratocytes, and induction of immune reactions.[14] Alternatively, non-viral vectors, such as lipids and nanoparticles, are generally safe but often found less efficient than their viral counterparts. For all this, adeno-associated viruses (AAV) stand as the most promising corneal delivery system at the moment, although they present a major disadvantage regarding low DNA packaging capacity, limiting some gene therapy approaches.[28] Furthermore, there remain other unanswered questions including the possibility for vector genome integration, especially in dividing cells; immunogenicity, whether humoral or cell mediated, possibly leading to a fast clearance of the viral vector; systemic biodistribution with off-

target expression; vector shedding and dissemination; and an overall compromised durability of the treatment effect persistence.[29] In this regard, further additional studies are required to better elucidate the widespread application of AAV vectors as a promising delivery system for corneal gene therapy.

Bench to Bedside – unmet challenges

Gene therapy for corneal dystrophies offers the promise to prevent or slow the progression of corneal diseases or provide a permanent cure. However, it is important to be realistic in terms of the challenges and hurdles that must be overcome to translate laboratory approaches into the clinical arena for both patients and corneal specialists. Although genetic analyses have provided important insights into the molecular architecture of corneal dystrophies, opening the path to promising preventive, diagnostic, and therapeutic strategies, they have also highlighted the difficulties in establishing genotype–phenotype correlations due to incomplete penetrance, phenotypic variability and genetic heterogeneity.[30] Furthermore, some diseases present heterogeneous complex inheritance, caused by the interaction between genetic and environmental factors, as it is the case of FECD, which interfere with the association of phenotypic traits and a specific genetic alteration.[31] In terms of methodology, most genetic screenings are focused on the study of coding exons and splicing boundaries of known candidate genes, and thus other alterations are inadequately assessed. Furthermore, relevant genes not yet connected to corneal dystrophies or other ocular diseases may be missing from these analyses.[32] Altogether, the identification of the pathogenic mutation underlying corneal dystrophy is not always achieved by genetic screening, and thus it stands as a basic limitation for gene therapy application.

Even when a disease-associated mutation is identified, different functional alterations may occur.[33] In consequence, developing personalized gene therapies may be only feasible when targeting commoner corneal dystrophies, like FECD resulting from a prevalent genetic defect.[17,23] Thus, mutation-independent gene therapies are required to increase clinical applicability.[22] Genome editing offers

promise but there are challenges to translate approaches into the clinical arena, including off-target effects and optimized delivery systems.[13] On the other hand, RNAi approaches might be more achievable in the short term, as shown by the phase III study of an ASO targeting insulin receptor substrate-1 expression as a treatment for keratitis-related progressive corneal neovascularization.[34] In addition, understanding the role of these genes in the development of corneal dystrophies is fundamental if gene therapy approaches are developed and introduced into the clinical arena. For example, the biology of *TGFBI* is not fully understood in the cornea and further studies are required.[35] In this sense, mouse models of corneal dystrophies are key to develop corneal gene therapies, although murine models of the *TGFBI* are limited to GCD2 (Arg124His)[36] and LCD1 (Arg124Cys).[37] In addition, murine phenotypic findings may differ from human phenotypes and may take significant time to develop, which can present challenges in the assessment of gene therapy correction strategies

Ultimately, it does not appear that the immediate future offers any genetically based therapeutic or preventative treatments for our corneal dystrophy patients. What is the reason for the relative lack of this research progress when contrasted with major advances in made in gene therapy for some retinal degenerations. Ultimately, corneal surgeons and their patients may be victims of our own treatment success. Individuals with some progressive retinal degenerations are programmed for progressive, profound visual loss with genetic therapy as the only potential treatment intervention to avoid relentless visual loss. Contrast this to the enlarging menu of highly successful corneal surgical procedures such as DALK, DSAEK and DMEK, which offer the possibility of visual improvement within weeks to months. With limited capital, should the ophthalmic community commit the required finances to the research required to make sufficient progress in the field of corneal dystrophies to eventually offer genetic interventions? We believe the answer is identical to what was expressed over a decade ago at the ARVO/Pfizer 2009 conference, when William Dupps MD explained “that inherited diseases such as the corneal dystrophies not only affect the patient, but also have effects that reach beyond the patient’s lifetime to innumerable offspring” and “research is critically important to those

who have the corneal dystrophies, because it offers the hope…of changing the future of innocent offspring by developing improved treatments, or even of prevention."[3]

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Legends

Figure 1 Fuchs endothelial corneal dystrophy. 53-year-old patient with cornea guttata and epithelial and stromal opacification in direct illumination by small slit lamp picture.

Figure 2 Schnyder corneal dystrophy. External photograph of the cornea of a 14- year- old male with a partial arc deposition of subepithelial crystals and uncorrected visual acuity of 20/20. Figure 9A reprinted from Weiss JS. Visual Morbidity in Thirty-Four Families With Schnyder Crystalline Corneal Dystrophy. Trans Am Ophthalmol Soc 2007;105:616-648.

Figure 3 Left photo-Lattice corneal dystrophy type 1 with genetic confirmation. Dots and paracentral lattice lines are seen in retroillumination. Figure 9D reprinted from Weiss JS, Moller HU, Aldave AJ et al. IC3D Classification of Corneal Dystrophies-Edition 2. Cornea. 2015 34:117-159. Right photo-Familial amyloidosis (Meretoja syndrome). Lattice lines are less numerous than in classic and variant LCD, start peripherally, and spread centrally
Figure 11 B reprinted from Weiss JS, Moller HU, Aldave AJ et al. IC3D Classification of Corneal Dystrophies-Edition 2. Cornea.2015 34:117-159.

Figure 4 Monoclonal gammopathy of undetermined significance. 72-year-old female patient: MGUS-induced paraproteinemic keratopathy in form of lattice lines in indirect illumination by dilated pupil.

Figure 5 Allele-specific RNA interference (ASP-RNAi) gene therap**y.** RNA interference (RNAi) can be achieved with a small interfering RNA (siRNA) designed to specifically target the mutant allele or mutation in an allele-specific manner (ASP-RNAi). When the siRNA binds to the mutant gene this leads to a loss of mutant protein expression. The normal copy of the gene is unaffected and so the normal protein is produced maintaining function. Created with BioRender.com.

Figure 6 CRISPR/Cas9 genome editing. Cas9 (CRISPR-associated protein 9) is a RNA guided enzyme that uses CRISPR (clustered regularly interspersed palindromic repeats) sequences as a guide to identify and cut specific strands of DNA that are complementary to the CRISPR sequence. Using site-specific RNA guide (sgRNA) sequences Cas9 can be directed to cut a target DNA sequence in the host genome with the caveat the target sequence is directly upstream of a protospacer adjacent motif (PAM). The cell will then attempt to repair the double strand break (DSB) in the target DNA sequence by either non-homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ is error-prone and can result in varying sizes of DNA insertions or deletions (indels) which can result in frameshift mutations and an absence of functional protein. NHEJ occurs during all stages of the cell cycle and so can be used in both dividing and non-dividing cells like the corneal endothelium. HDR is more precise but is limited to dividing cells. HDR uses a homologous repair template, either the homologous chromosome or an exogenous homologous repair template in high concentration. The exogenous homologous repair template can be used to correct point mutations. Adapted from "CRISPR/Cas9 Gene Editing", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.