Review Article - Narrative Review

Taiwan J Ophthalmol 2023;13: 467-478

Access this article online

Quick Response Code:

Website:
http://journals.lww.com/TJOP

DOI:
10.4103/tjo.TJO-D-23-00140

New horizons in aniridia management: Clinical insights and therapeutic advances

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Abstract:

Congenital aniridia is a rare genetic eye disorder characterized by the complete or partial absence of the iris from birth. Various theories and animal models have been proposed to understand and explain the pathogenesis of aniridia. In the majority of cases, aniridia is caused by a mutation in the *PAX6* gene, which affects multiple structures within the eye. Treating these ocular complications is challenging and carries a high risk of side effects. However, emerging approaches for the treatment of aniridia-associated keratopathy, iris abnormalities, cataract abnormalities, and foveal hypoplasia show promise for improved outcomes. Genetic counseling plays a very important role to make informed choices. We also provide an overview of the newer diagnostic and therapeutic approaches such as next generation sequencing, gene therapy, *in vivo* silencing, and miRNA modulation.

Keywords:

Congenital aniridia, PAX6 gene mutations, Aniridia-associated Keratopathy (AAK), clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)

Introduction

Aniridia is a rare, site threatening disorder characterized by the underdevelopment or absence of the iris and fovea, leading to decreased visual acuity and involuntary eye movements. This condition typically becomes apparent during early infancy. The majority of cases of aniridia are linked to inherited mutations or deletions in the *PAX6* gene, which typically follow a dominant inheritance pattern.

Anatomy of Iris

Iris is the anterior most portion of the uveal tract and separates the aqueous compartment into the anterior and posterior chambers. Iris diaphragm is the thickest at the collarette and thinnest at the root of iris. The layers of the iris can mainly be divided into anterior limiting layer, the stroma and the posterior layers consisting of pigment epithelium and the dilator muscle.[1]

The arrangements of fibroblasts, melanocytes, and collagen are denser in the anterior limiting layer compared to the stroma. The crypts of iris cause the irregular surface of iris and are more prominent in the pupillary zone and toward the root of iris.[1] The anterior limiting layer terminates abruptly toward the root of iris. The iris processes continue toward the Schwalbe's line.[2]

The stroma of the iris is made up of both pigmented and nonpigmented cells, along with extracellular materials such as collagen fibrils embedded in a matrix of mucopolysaccharides. These fibrils are usually organized in columns or bundles around the nerves and blood vessels in the iris stroma.[3] Sphincter muscle is a smooth muscle located in the pupillary area of the stroma measuring around 1 millimeter in width.[1]

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Submission: 14-09-2023
Accepted: 11-10-2023
Published: 20-12-2023

How to cite this article: Gour A, Tibrewal S, Garg A, Vohra M, Ratna R, Sangwan VS. New horizons in aniridia management: Clinical insights and therapeutic advances. Taiwan J Ophthalmol 2023;13:467-78.

The iris is supplied by the major arterial circle in the ciliary body. The arteries and veins form the bulk of the iris stroma. The iris is drained by the collector channels traversing the ciliary body and finally into the vortex system of the ciliary plexus.[1]

The posterior layer is composed of the anterior nonpigmented and posterior pigmented epithelium. The dilator muscle is situated along the posterior boundary of iris stroma and peripheral to iris sphincter.[1]

Embryology of Iris

The development of iris starts after the closure of embryonic fissure and is completed by six weeks of gestation.[1] The pigment epithelium is neuroectodermal in origin. The outer wall of the optic cup gives rise to anterior pigment epithelium and inner wall gives rise to the posterior pigment epithelium.[4]

The dilator and sphincter muscles of the iris are formed by the neuroectodermal cells of optic cup. They are formed between 6 and 8 months of gestation.[5]

The pupillary membrane and iris stroma are mesenchymal in origin, arising from the third wave of migrating neural crest cells.[6] During the second month of gestation, the anterior rim of the optic cup develops a thin layer around the anterior surface of the lens. The central part of this layer becomes the pupillary membrane which later, regresses to form the pupil.[5] The mesenchymal cells and stromal melanocytes migrate along the anterior border of the iris epithelium forming the iris stroma and blood vessels.[7] *PAX6* is expressed in a transitory manner and at lower concentrations than its expression by ectoderm during morphogenesis of this part of the iris.[4]

Theories Explaining the Pathogenesis of Aniridia

Ectodermal theory

Failure in the development of the rim of the optic vesicle during the 12th–14th week of gestation results in aniridia. It is commonly associated with other ectodermal defects such as anomalies in the retina, absence of fovea, and absence of pupillary musculature.[8]

Mesodermal theory

In the second month of gestation, abnormal development of mesoderm could potentially lead to aniridia in two different ways. First, if the mesodermal wedge that usually grows between the surface ectoderm and rim of the optic cup only appears as a narrow band at the periphery of the anterior chamber, it would prevent the ectoderm from growing at the front. Second, if the capsulopupillary vessels of the tunica vasculosa lentis do not atrophy, it would hinder growth of the optic cup rim and cause a hypoplastic iris. However, these mesodermal theories do not provide an explanation for the frequent occurrence of aniridia alongside neuroectodermal abnormalities such as foveal hypoplasia.[8]

Environmental factors

Aniridia has been proposed to be associated with maternal Vitamin A deficiency.[1]

Histology of Aniridia

Severe hypoplasia of the iris is the usual abnormality noted histologically. The anterior border of iris stump consists of closely packed melanocytes. This forms a flatter iris surface with fewer crypts. The pupil border may be covered by several redundant layers of pigment epithelium.[1]

Vessels of the iris are prominent, but the dilator and sphincter muscles of the iris are rarely visualized. The anterior chamber angle is noted to be normal usually. Either incomplete cleavage or anomalous anterior chamber angle may be noted. In incomplete cleavage, the position of the angle recess is normal; however, it is filled with loose mesenchymal tissue and trabecular meshwork and Schlemm's canal are unidentifiable.[1] Anomalous angle has been associated with partial deletion of chromosome number 11 and showcase a more severe developmental abnormality.[9] The internal sulcus may either be filled with iris tissue instead of TM or may be completely absent with the root of iris inserted posteriorly over the ciliary body.[1]

Epidemiology

The prevalence of aniridia has been reported to be 1 in 40,000–100,000, with no gender predilection.[10]

Genetic Aspects of Aniridia

PAX6[11] was identified as a key aniridia-causing gene during the search for the genetic causation of WAGR (Wilm's tumor, aniridia, genitourinary anomalies, and intellectual disability) syndrome which is caused by hemizygous deletions of 11p13. The *PAX6* gene codes for one of the key embryonic transcription factors and serves as a master regulator of eye and CNS morphogenesis in all species of bilaterian animals.[12] The *PAX6* protein consists of two highly conserved DNA-binding domains: paired and homeobox. To ensure specific regulation of target genes, the domains are able to bind different DNA motifs either independently or in cooperation or even antagonizing in different cell contexts. *PAX6* has a complex temporal and tissue-specific expression pattern. Either excessive or insufficient expression levels,

as well as a misbalanced ratio of expressed transcript variants, lead to the disturbance of embryogenesis and organogenesis. Homozygous or compound heterozygous mutations in the *PAX6* gene are known to be lethal. Most heterozygous mutations lead to the loss of function of mutated PAX6 allele (haploinsufficiency). These variations in *PAX6* result in several phenotypes. The most frequent phenotype is congenital aniridia (>90%), which is characterized by damage to various eye structures often accompanied by morphological and functional disorders of other organs and systems.

Mutations in *PAX6* gene or 11p13 chromosomal aberrations[13] around the upstream and downstream coding and/or regulatory regions of the *PAX6* gene have been greatly studied. *PAX6* gene follows an autosomal dominant pattern of inheritance and can be passed on 50% times from one affected parent of either sex.

Suggestions about gene dosage effect by haploinsufficiency of *PAX6* and localization of *PAX6* transcripts in the various parts of the eye and body leading to multisystemic malformations were made by Glaser *et al.* in 1994. Gupta *et al*., in a study of 12 probands, reported a correlation between certain aspects of aniridia phenotype, such as congenital cataract with the presence of mutations on the paired and C-terminal proline-serine-threonine–rich domain of the *PAX6* protein. A large cohort study of 43 patients[14] with *PAX6* mutations also reported a severity correlation between loss-of-function and C-terminal extension mutations with severe phenotypes such as foveal hypoplasia, marked iris anomalies, and severe visual impairment when compared with missense mutations. A similar observation was also made by Kit *et al*. in a group of 86 affected individuals with the missense mutation subgroup having the mildest phenotype and maintaining better visual acuity.[15]

Vasilyeva *et al*.[13] on analyzing the genetic profile of 155 aniridia patients observed that subjects with 3'-cis-Regulatory region deletions had a milder aniridia phenotype without keratopathy, nystagmus, or foveal hypoplasia. However, no other significant phenotypic association was observed in other patients with rearrangements involving 11p13 when compared with point pathogenic variants in the *PAX6* gene. A severe phenotypic outcome was associated with missense mutations and genetic variants disrupting splicing resembling loss-of-function mutations. They also noted multiple eye malformations in aniridia for patients that were *PAX6* mutation positive.

On the other hand, Yokoi *et al*. observed no clear correlation between genotype and phenotype after studying 5 familial and 16 sporadic *PAX6*-positive aniridia cases.[16] Even in members of the same family who had the same mutation, the phenotype of aniridia was partially different with differences in the degree and appearances of corneal opacity, iris remnant, cataract, foveal hypoplasia, and optic nerve hypoplasia. However, all ocular findings were almost completely symmetrical in both eyes of each patient.

Due to limited access to the human eye for experimentation, animal and cellular disease models[17] have been crucial in identifying the genetics and pathophysiology leading to aniridia. Different vertebrate species share similar structure and function. Hence, such animal models allow us to understand molecular pathways involved in disease progression and consider genotype–phenotype correlations. The discovery of homologous genes to *PAX6* in Drosophila, Xenopus, zebrafish, and mice has enabled the study of this disease, the pathway affected by PAX6 deficiency, and the development of therapeutic models.[18]

Mouse models

It is the striking similarity between the mouse eye and human eye in terms of physiology, anatomy, and development that makes mice a good model to study human eye diseases, especially for aniridia, as human *PAX6* and mouse *Pax6* are homologous and translate into the same protein sequence.[18] *Pax6* variations in mice are either spontaneous, induced by chemicals or radiation, or targeted mutations. The first of its kind *Sey* mouse contained a spontaneous point mutation c.622G>T p.(Gly208*) that results in a premature termination codon (PTC). The effect on the phenotype and its variability with Homozygous $Pax6_{Sey/Sey}$ mice and heterozygous $Pax6_{Sey/+}$ mice was studied. It was observed that while the homozygous variation was lethal, the heterozygous[17] variant produced an array of dysmorphologies such as iris hypoplasia, abnormal lens morphology, cataracts, corneal opacification, and incomplete separation of lens from the cornea, all of which are also found in human aniridia patients. Other chemically induced missense or nonsense variations have also been introduced into mice such as $Pax6_{Sey\text{-}Neu}$ and the $Pax6_{Neu}$ series to quantify Pax6 protein levels that are translated from the corrected mutant allele following gene editing. *PAX6* gene deletion models $Pax6_{Sey\text{-}Dey}$, $Pax6_{Sey\text{-}H}$, and $Pax6_{tm1Pgr}$ (Pax6lacZ) contain whole gene deletions have helped uncover differences in the *PAX6/Pax6*- controlled mechanisms between humans and mice. For example, Voskresenskaya *et al.* found that in humans, unlike mice, *PAX6* pathogenic variants do not delay lens placode development or alternatively, it recovers from the delay.[19]

These studies have contributed to our knowledge of aniridia in helping us understand the pathophysiology of

aniridia and testing potential therapeutics. For example, understanding the dosage effect of PAX6 has helped develop dosage targeted therapies.[20] Promising results have been demonstrated by nonsense[21] suppression drugs by inhibiting disease progression and rescuing corneal, lens, and retinal malformations postnatally. Upregulation of Pax6 leading to rescue from corneal phenotypes in aniridia has also been shown by inhibition of mitogen-activated protein kinase through small molecule drugs in $Pax6_{Sey\text{-}Neu/+}$ mice.

Zebrafish models

The significant genomic similarity between humans and zebrafish makes it a good model to study vertebrate disease with 84% of human disease-causing genes having a zebrafish counterpart.[22] The *sunrise* (*sri*) mutant, a mutant that carries a leucine to proline missense mutation c. 770T > C, p.(Leu244Pro) in the *pax6b* homeodomain gene in zebrafish was produced from a batch of large-scale chemical mutagenesis using ENU. *Sunrise* is the most widely explored zebrafish model with a *pax6b* mutation and exhibits aniridia-like phenotypes, for example, abnormal lens and corneal structure, thick cornea, iris hypoplasia, retinal malformations, shallower anterior chamber, and a smaller eye.

Human cellular models

Patient-derived cells, thereupon, help understand disease mechanisms and carry out drug screening. Such models also help reduce the number of animals used in biomedical preclinical experiments and raise the ethical standard of practice.[23]

Ocular findings, pathogenesis, diagnostic investigations, and treatment of aniridia-associated conditions

Dry eye disease

A high prevalence of DED has been reported in patients with AAK.[24,25] The mechanism for dry eye in patients with aniridia has been attributed to a low production of tears, a poor quality of the tear film, and stenotic Meibomian gland orifices. Studies showed that aniridia patients have more severe DED than healthy individuals, including elevated tear film osmolarity and increased atrophy of the Meibomian glands.[26] Thus, Meibomian gland dysfunction (MGD) plays a significant role in developing DED in aniridia. It has also been found that aniridia subjects have a higher corneal vital staining score, which was positively correlated with the stage of aniridia-associated keratopathy (AAK) and negatively correlated with corneal sensitivity. This indicates that corneal disease may trigger the development of DED in aniridia patients and vice versa, where DED could cause or worsen AAK.[27]

An imbalance between pro- and anti-inflammatory cytokines in the tear fluid with a disproportionate increase in proinflammatory cytokines has also been shown.[28]

Aniridia-associated keratopathy

The prevalence of AAK is varied [Figure 1]. It is noted to be in approximately 80% of the diseased population in some studies,[29,30] while a study by Aniridia Foundation International reported AA in around 45%.[25]

In individuals with congenital aniridia, corneal abnormalities can take two forms: congenital central corneal opacity (CCO), which presents as dense central corneal opacification from birth, or aniridia-associated keratopathy (AAK), where the central cornea is clear at birth, but lesions start in the corneal periphery and progress to the central cornea during teenage and early twenties. AAK is caused by a deficiency in the limbal stem cell niche and is characterized by conjunctival tissue overriding the limbus. Unilateral cases are more common in CCO, and CCO is reported to be significantly associated with a higher rate of glaucoma than AAK. In addition, the visual outcome was worse in patients with CCO than in those with AAK due to the higher prevalence of amblyopia in these patients.[31]

Several classification systems have been used for the grading of AAK. A 5-point classification of AAK for clinical evaluation and research work has been used widely as it includes the distinction between the lower grades of AAK.[32] Another classification and staging of limbal stem cell deficiency have been proposed. However, it does not recognize the precise pattern and progression of keratopathy in aniridia.[33]

The 5-point grading scale includes Grade 0, representing a healthy corneal limbus on slit lamp examination. As the grade of AAK progresses, there is an increasing breakdown of the limbal border and encroachment of the conjunctival tissue onto the cornea. Grade 4, is the advanced, end-stage AAK characterized by total corneal conjunctivalization and vascularization.[32]

Treatment for aniridia-associated keratopathy includes conservative and surgical management of the condition

Conservative treatment at early stages involves the use of preservative-free artificial tear fluid containing

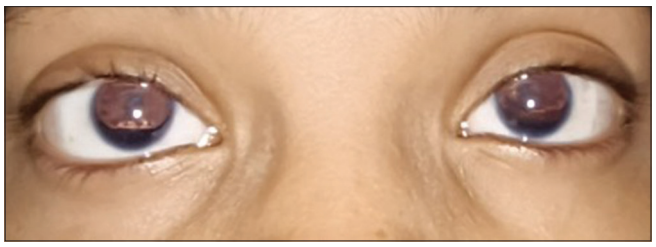

Figure 1: Aniridia-associated keratopathy

hyaluronic acid and dexpanthenol.[34] A reduction in epiphora symptoms, photophobia, foreign-body sensation, and signs such as improved tear film stability have been reported using autologous serum eye drops.[35]

Amniotic membrane transplantation: The amniotic membrane has growth factors that aid in the healing of the ocular surface. Research has shown that early amniotic membrane transplantation in patients with moderate limbal stem cell deficiency causes stabilization of the ocular surface and improved visual acuity.[36] However, recurrence of corneal epithelial defects, ulceration, and epithelial cell metaplasia has been demonstrated.[37]

In the past, AAK was commonly treated with penetrating or lamellar keratoplasty alone, but this approach led to a high recurrence of keratopathy due to underlying limbal stem cell deficiency. Therefore, performing keratoplasty without addressing the limbal stem cell deficiency will only provide a temporary improvement in AAK.[27]

Limbal stem cell transplantation is commonly preferred in grade 2 and 3 of AAK to treat the underlying stem cell deficiency and prevent stromal scarring. This can be done through living-related conjunctival-limbal allograft (lrCLAL) transplantation or keratolimbal allograft (KLAL) transplantation. Currently, lr-CLAL is preferred due to its lower risk of failure/rejection when proper immunosuppression is administered. In cases where stromal scarring has already occurred, penetrating keratoplasty can be performed following LSCT to remove the scarring.[34]

If the risk of limbal graft failure is low, limbal stem cell transplantation followed by PKP is the preferred optical procedure over KPro. Recent studies have shown that factors such as ocular surface inflammation, severe dry eye, ocular surface exposure, and the inability to tolerate long-term systemic immunosuppression are associated with an increased risk of graft failure.[34,38]

In cases with a high risk of limbal graft failure or the patient cannot tolerate systemic immunosuppression due to their age or systemic co-morbidities, the Boston Keratoprosthesis (KPro) type 1 is preferred over LSCT. Patients who have previously undergone graft failures (PKP or LSCT) and have already been immunologically sensitized are at a high risk of LSCT failure.

For end-stage AAK, KPro is generally preferred since LSCT alone cannot restore vision and additional keratoplasty procedures may increase the risk of rejection/failure. However, LSCT combined with PKP may be considered as an alternative in patients without prior transplants or tube shunts, as tube shunts can lead to recurrent endothelial failure. Studies have demonstrated good success rates and long-term retention of primary KPro in patients with limbal stem cell deficiency, with approximately 80% retention and good visual acuity at 3 to 5 years. Primary KPro implantation has shown better long-term visual outcomes compared to KPro implantation after corneal transplantation.[39]

A recently published study investigates the role of KeraKlear nonpenetrating artificial corneas as an alternative to limbal stem cell transplantation and penetrating keratoplasties in two patients. These patients demonstrated an improved visual acuity. However, complications such as corneal thinning, neovascularization, and retroprosthetic opacity were reported.[40] Thus, more studies on a larger cohort population need to be performed to evaluate its efficacy.

Iris abnormalities and their management

Iris hypoplasia is a universal finding in aniridia and defines the condition [Figure 2]. The amount of absent iris tissue varies from partial to total. Even in apparent total aniridia, in most cases, a stump of iris tissue can be visualised during gonioscopy or on histology.[41] The iris tissue deficiency *per se* does not lead to any loss in vision. However, it is responsible for some of the photophobia and glare experienced by these patients. The absence of iris can also lead to a loss of depth of focus and cosmetic blemish. The deficient iris can be replaced by prosthetic devices through surgical correction. Different types of iris prosthetic devices are available and can be customized to each case.[42-44] The capsular tension-based devices such as aniridia ring segments and rings (Morcher GMBH, Stuttgart, Germany) can be implanted into the capsular bag during cataract surgery in aniridia patients.[45,46] The ring segments are used in partial aniridia wherein the segment is aligned in the capsular bag in such a way that it underlies the iris defect. The aniridia rings are used in total aniridia and

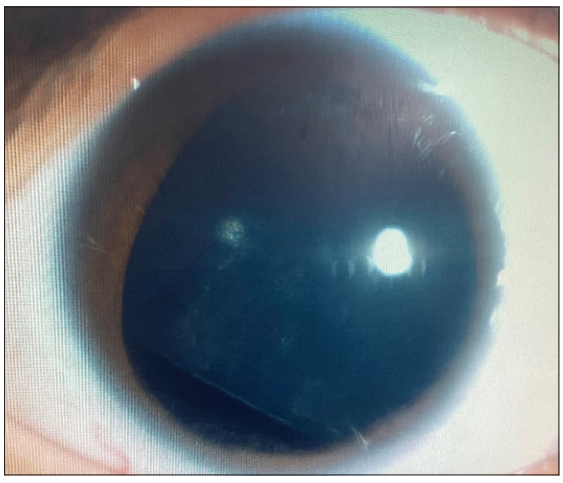

Figure 2: Iris hypoplasia in aniridia

consist of a pair of capsular tension rings with multiple small opaque segments placed equidistant to each other. The two rings are implanted in the bag and aligned to interdigitate the segments and form a complete opaque ring of the prosthetic device. These devices can be combined with in-the-bag implantation of a foldable intraocular lens. These rings have the disadvantage of being available in only black color and hence may not be acceptable in unilateral cases. Furthermore, they are slightly fragile and prone to fracture during implantation.[46] These rings should not be implanted if the capsular bag is compromised, in which case they can migrate to the sulcus and damage the anterior chamber angle. A device with two orthogonal segments with a flexible joint (Ophtec BV, Groningen, The Netherlands) is also available, which is sturdier, does not require interdigitating of two rings but needs a larger wound for implantation.[47]

Small iris defects may be amenable to direct closure using 10-0 polypropylene sutures. The combined iris-lens implant is the second type of iris prosthetic device. It consists of a polymethylmethacrylate intraocular lens implant with a clear central optical zone and opaque black-colored peripheral zone corresponding to the iris. These lenses are large (10 mm) and require a large wound for insertion. They cannot be implanted in the bag due to their large size and are provided with fixation loops on the haptics for sulcus fixation.

The third type of iris prosthetic device is the hydrophobic silicone elastomer iris diaphragm (Artificiallaris [HumanOptics, Germany] and CustomFlex™artificial iris [USA]).[48,49] This is a foldable device which can be inserted from a phacoemulsification wound and can be customized to match the iris color of the contralateral eye. It can be implanted in the bag, sutured to the sulcus, or sutured to the anterior surface of the sulcus-fixated intraocular lens. It may be possible to place it in the sulcus in cases where reasonable amount of iris tissue is present.[49] The iris diaphragm should be avoided in phakic cases, as it can lead to cataract formation or corneal decompensation.[43]

A judicious use of all types of iris prosthetic devices is recommended. These devices are not devoid of complications. Intraoperative complications such as capsular tear, implant fracture and hyphema, and postoperative complications such as prolonged inflammation, anterior migration of the implant, progression of the corneal epithelial disease, progression of pre-existing glaucoma, or new-onset glaucoma have been reported in the literature.[43,44] Furthermore, photophobia and glare in these patients is also a result of the coexisting foveal hypoplasia and not entirely due to the absent iris.

Routinely available colored contact lenses are best avoided in aniridia due to the risk of aniridia-associated keratopathy. However, scleral contact lenses may be a better option. A smart scleral contact lens with liquid crystal-based artificial iris has been developed which provides a promising option in patients with aniridia.[50]

Lenticular abnormalities and their management [Figure 3]

Cataract is the most common lenticular abnormality in aniridia in 40%–82% of cases.[51-54] Other rarely seen lenticular abnormalities are lens subluxation, lens coloboma, microspherophakia, and posterior lenticonus. The onset of lenticular opacification happens at any age and usually accelerates in the second decade of life.[55] The typical aniridic cataract begins as a posterior polar or posterior subcapsular cataract with radial spokes in the mid periphery, which gradually progresses to total opacification. Other morphological forms of cataract such as nuclear cataracts, partially absorbed membranous cataract, hypermature cataract with subcapsular edema, and anterior polar cataract have also been reported.

A visually significant lenticular opacity warrants surgical removal and intraocular lens implantation. Cataract surgery in such cases can be challenging owing to the poor ocular surface, varying grades of corneal opacification, fragile anterior and posterior capsule and the deficient iris. An advanced grade of aniridia-associated keratopathy obscuring visualization of the anterior chamber and lens may warrant limbal stem cell transplantation as the first step. For the cataract surgery, a corneal or limbal incision may be preferred when small phacoemulsification wounds are desired. A scleral tunnel should be avoided, considering that these patients may require glaucoma surgeries in future. However, when large wounds are required, a temporal scleral wound may be fashioned to avoid damaging

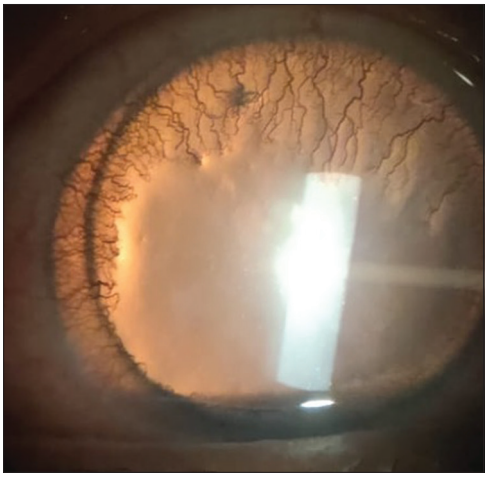

Figure 3: Limbal stem cell deficiency, iris hypoplasia, and lenticular abnormalities in Aniridia

a large area of limbal stem cells. The anterior capsule is known to be fragile due to alterations in basement membrane constituents in aniridia. This makes anterior capsulorhexis challenging. It should be performed with utmost care so that a continuous curvilinear opening of adequate size is created. A compromised capsulorhexis will preclude implantation of the intraocular lens in the bag, which is preferred over sulcus or scleral fixated IOL to reduce the risk of postoperative glaucoma. Capsular staining with trypan blue and use of cohesive viscoelastic material helps construct the capsulorhexis. In children younger than 6 years of age, a primary posterior capsulotomy (PPC) and anterior vitrectomy should be performed to prevent visual axis opacification in the follow-up period. A PPC may be required in older children who are either uncooperative or have a significant nystagmus and may not allow YAG capsulotomy in future. Iris reconstructive procedures may be combined with cataract surgery in selected cases as described above.

Cataract surgery in aniridia is not free from complications. These are increased postoperative inflammation, uveitis, glaucoma, exacerbation of keratopathy due to limbal stem cell loss, endothelial damage, and cystoid macular edema. Dye to the profibrotic nature of capsule in these cases, the capsular bag may contract with time and lead to decentration of the IOL and displacement of the aniridia rings. Thus, cataract surgery is indicated only when the lenticular opacification is significant enough to affect the vision in aniridia.

Optic nerve abnormalities

The most common refractive error seen in aniridia is myopia. Myopic shift and an increase in astigmatism have been noted with age. The optic nerve may be abnormal in patients with aniridia but not so commonly as the fovea. The optic nerve abnormalities seen are optic nerve hypoplasia (2%–30%), optic disc aplasia, optic disc pit, morning glory disc, optic disc pallor, and glaucomatous optic atrophy. Rarely, Meibomian gland dysfunction and ptosis have already been reported.

Foveal hypoplasia and nystagmus

Foveal hypoplasia is the most common ocular comorbidity in aniridia following iris hypoplasia. Several studies have noted it up to 90% of the cases. It is present since birth and is often associated with nystagmus (55%–75%). Foveal hypoplasia is characterized by the absence of the foveal pit and the foveal avascular zone. The retinal vessels are seen coursing throughout the macula, and autofluorescence can detect the absent hypo fluorescence at the region of the fovea. The foveal contour and the thickness of the retinal layers at the fovea determine the structural grading of the hypoplasia, as revealed by optical coherence tomography (OCT). Rufai *et al*. combined the OCT-based grading system for foveal hypoplasia proposed by Thomas *et al*.[56] and Wilk *et al*.[57] and referred it to the Leicester Grading System for foveal hypoplasia. They found that the structural grading was a better predictor of future visual acuity in children with nystagmus and was superior to the preferential-looking tests. Casas-Llera *et al*. also showed that outer segment lengthening correlated with better vision in patients with aniridia.[58] The grading is based on four salient features seen on the OCT images: presence/absence of the foveal pit, presence/absence of extrusion of the plexiform layers, presence/absence of outer nuclear layer widening, and presence/absence of outer segment lengthening. In the most severe grade of foveal hypoplasia, all four features are absent. Thus, in severe grades, the foveal thickness increases as the foveal pit becomes shallower, and the plexiform layers are not extruded from the fovea. At the same time, the outer nuclear layer, outer segment, and Henle's layer become thinner. The changes can be seen even in the choroid in the form of thinning of the subfoveal and parafoveal choroid.

Electroretinogram is abnormal in aniridia. The oscillatory potentials are reduced the most, followed by b wave and a wave. Both rods and cones are shown to be affected, and thus, changes can be seen in both multifocal and full-field ERG. Dangremond *et al*. demonstrated in six aniridia patients that the amplitudes of rings 1–3 were reduced while those in rings 4–5 were increased in the multifocal ERG.[59] The higher amplitude in rings 4 and 5 correlated with poorer visual acuity. The amplitude of waves in the full field ERG were similar to controls, but the latency of scotopic and photopic b wave, scotopic a wave and 30Hz flicker were prolonged as compared to controls. Tremblay *et al*. correlated the ERG changes to specific *PAX6* mutations and found that mutations affecting the paired domain of the PAX6 protein had the biggest impact on the electroretinogram amplitudes.[60] The retina is also hypopigmented in most aniridic eyes, which could be attributed to the role of *PAX6* in retinal pigment epithelium differentiation.

Systemic manifestations of aniridia

a. Brain anomalies: A high percentage of patients with *PAX6* mutation have brain anomalies, including excessive cortical thinning; changes in gray matter in the cerebellum; temporal and occipital lobes; hypoplasia of optic chiasma, anterior and posterior commissure, and corpus callosum. These patients may also have an absent or hypoplastic pineal gland.[27] MRI may reveal alteration in the white matter structure and posterior visual pathways.[61] A decreased activation of the fronto-striato-thalamic system and activation of the supplementary occipital, frontal, and medial temporal regions have been

demonstrated on the MRI indicating the specificity and refinement in such patients.[62]

b. Auditory deficits: children with aniridia associated with *PAX6* mutation may have difficulty in localizing and comprehending speech, especially in noisy surroundings. In addition, verbal working memory may be impacted. While audiograms display normal findings, interhemispheric pathway abnormalities are displayed on the MRI. Electroencephalography abnormalities have also been demonstrated in such patients.[63]
c. Hormonal changes: Diabetes mellitus of varying degrees is a frequently reported occurrence in patients with aniridia. Mice with *PAX6* mutations have been shown to have decreased glucagon and insulin production due to a reduction in endocrine cells. As a result, such children suffer from morbid obesity. In addition, a significantly lower melatonin secretion with higher sleep disturbances has been reported in such patients, while narcolepsy is also reported. MRI scans may reveal hypoplasia of the pineal gland.[27]

Association with other syndromes

a. WAGR syndrome (Wilm's tumor, Aniridia, genitourinary abnormalities, and mental retardation) is the most common syndrome associated with aniridia. The diagnosis of WAGR syndrome is made when aniridia, along with at least one of the conditions of the syndrome, is present. Deletion of *PAX6* and *WT1* gene on chromosome 11p is known as "WAGR deletion." Wilm's tumor is usually seen in patients before 2–3 years of age. Such patients also have associated craniofacial dysmorphism[64]
b. WAGRO syndrome (WAGR syndrome associated with obesity): this is associated with the deletion of the obesity gene[64]
c. Gillespie's syndrome: a rare variant form of aniridia. The classical triad includes partial aniridia, nonprogressive cerebellar ataxia, and mental retardation. *ITPR1* is the associated causative gene. Other ocular findings include hypertelorism, nystagmus, strabismus, ptosis, corectopia, optic disc hypoplasia, foveal hypoplasia, and pigmentary retinopathy.[4]

Role of Genetic Counseling

Genetic counseling refers to guidance relating to genetic disorders that a specialized health-care professional (genetic counselor) provides to an individual or family. It includes a discussion about how a genetic condition could affect an individual or family and/or the interpretation of genetic tests designed to help estimate the risk of a disease. The genetic counselor conveys information to address the concerns of the individual or family, helps them make an informed decision about their medical situation, and provides psychological counselling to help them adapt to their condition or risk.

Isolated aniridia is inherited in an autosomal dominant manner. Approximately 70% of individuals diagnosed with isolated aniridia have an affected parent (i.e., familial aniridia), and the rest are due to *de novo* mutations in the gene.[65] If an individual tests positive for variations in *PAX6*, their parents should undergo clinical screening to eliminate the chances of minor degrees of iris hypoplasia or reduced visual acuity caused by foveal hypoplasia. The family history of some individuals due to the same reason may appear negative. Therefore, an adverse family history cannot be confirmed unless appropriate clinical evaluation and/or molecular genetic testing has been performed on the proband's parents. In a few exceptional cases, negative molecular screening results of parents could be due to germline mosaicism in one partner. The family should be made aware of this possibility during pretest counseling to caution about recurrence risk and limitations of genetic tests. In some scenarios, multiple siblings affected by unaffected parents could also be explained by alternate paternity, maternity, or undisclosed adoption and should be explored in a genetic counseling session. In most cases, when the parents are clinically unaffected, the risk to the proband's siblings is low. Individuals with the genotypically mosaic distribution of mutated versus wildtype *PAX6* in their leukocytes have a relatively lower risk of transmission to offsprings. Genetic counseling becomes important for affected individuals at premarital age to make informed choices regarding marriage, conception, prenatal testing options, and preimplantation genetic testing.

Innovative Diagnostic Strategies

The introduction of next-generation sequencing (NGS) methods in exhaustive molecular testing has led to significant advancements in recent years. These advancements have enabled the discovery of new genes and various mutations linked to several inherited disorders, such as the spectrum of ASD disorders and congenital aniridia.[66] Before the advent of NGS, the molecular diagnostic testing strategies for *PAX6* involved a two-step approach. Initially, it involved screening for large deletions or chromosomal rearrangements through methods such as karyotype analysis, microarray-based comparative genomic hybridization, and fluorescence *in situ* hybridization. The second step involved detecting small intragenic sequence variants through PCR-based Sanger sequencing of *PAX6*'s entire coding region and intron-exon boundaries. While targeted next-generation sequencing helps in detecting variants at various genomic scales in a single assay, which includes the detection of single nucleotide variants and copy number variations.

Due to these advantages, NGS is being exploited in many countries for detecting rare disorders.

Recently, a diagnostic genetic testing using Illumina NextSeq 500 platform with whole-genome sequencing (WGS) approach on Illumina Novaseq600 platform was tested in 450 cases of diverse ASD disorders including congenital aniridia. In 230 unrelated families, pathogenic and likely pathogenic variants in *PAX6* were identified. The classic mode of presentation of this disease was reversed with 40% of the 230 PAX6 positive families having a family history and 60% being sporadic cases. The use of NGS demonstrated that, with the exception of two cases featuring mosaic alleles, all *PAX6* variants were germline, thereby highlighting the capability of NGS to precisely assess the degree of mosaicism with de novo variants (Valleix *et al*., VI European Aniridia Conference, June 2022). Besides all this, NGS also revealed single nucleotide, structural variants, and copy number variations in *PAX6*. In cases where families were still genetically unexplained following NGS analysis, parent-offspring trios underwent paired-end WGS in the clinical setting, and it was found that structural variants physically separated *PAX6* and *PITX2* genes from their downstream and upstream regulatory elements, leading to complete loss of expression by positional effect. Thus, the findings of this study provide evidence that a screening strategy combining NGS and WGS enhances the diagnostic yield of aniridia and enables the identification of variants that may not have been covered by conventional diagnostic technology platforms.

Innovative Therapeutic Approaches

Individuals with aniridia typically have impaired vision from birth. As they reach young adulthood, they may become legally blind due to progressive complications that can cause sight loss, such as glaucoma, cataracts, and corneal opacification. Despite medical and surgical interventions, there are currently no long-term treatments or cures available for aniridia. However, some secondary ocular abnormalities can be partially managed. Surgical procedures on aniridia eyes may exacerbate aniridia-related keratopathy and further deteriorate vision. Therefore, there is a pressing need for new therapeutic approaches to address aniridia. Some of the new approaches that are being considered are gene therapy (adeno-associated virus (AAV) delivery, *in vivo* gene editing through clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) and nonsense mutation suppression), and drug repurposing.

Gene therapy could be a potential method for treating aniridia, which involves introducing genetic material into cells to create the necessary protein (augmentation) or correct the faulty gene (gene editing). However, it is important to note that over-expression of PAX can have detrimental effects on normal eye development.[67] Therefore, to achieve successful therapeutic outcomes, precise regulation of *PAX6* would be crucial. Thus, in applications where restricted expression is desired, such as targeting transcription to a specific tissue or limiting it to particular cells, a toolbox of specific promoters would be advantageously known as "Mini-promoters." Elizabeth Simpson's team has designed mini-promoters for PAX-6, with vectors containing small specific regulatory regions of the *PAX6* gene.[68] By using cell-specific mini-promoters, very precise regulation of PAX6 would be done, and currently, research groups are targeting cornea cells where they can be used to achieve therapeutic success.[68]

In vivo gene silencing

One potential approach to treating aniridia is targeted CRISPR-based genome editing, where CRISPR can be used to revert a mutation to the correct sequence in the genome, thereby allowing for the normal regulation of expression levels and timing in appropriate cell types.[69] In the past, there is evidence of successful delivery of CRISPR/CRISPR-associated protein 9 (Cas9) components through viral vectors to rodent and nonhuman primate eyes.[70,71] Hence, efforts are being made to increase the expression of *PAX6* through a CRISPR-based gene-editing strategy to improve the structure and function of the eye and also rescue the mutant phenotype in the *in vivo* model. Elizabeth and Mirjalili group[72] have successfully used CRISPR/Cas9 technology to produce a new mouse model of aniridia, closer to the human phenotype and also shown both *in vitro* (by electroporation in embryonic stem cells) and *in vivo* (by microinjection into zygotes) the germline correction of the mutation with an average success rate of 34.8% and 25%, respectively.

miRNA modulation

miRNAs are small noncoding RNAs that posttranscriptionally regulate gene expression and protein levels and also play a role in maintaining normal corneal homeostasis, in age-related macular degeneration (AMD) and diabetic retinopathy (DR), in the regulation of angiogenesis, oxidative stress, immune response, and inflammation. Latta *et al*. have reported dyregulated levels of miRNAs in bulbar conjunctival cells of aniridia and healthy subjects.[73] The most important downregulated miRNA found in aniridia patients was mainly related to controlling neovascularization and wound healing (miR-204-5p). When related clinically, it was found downregulation of miR-204-5p was associated with highly neovascularized corneas observed in severe AAK. Thus, it was hypothesized, downregulation of miR-204-5p promotes proliferation and migration of conjunctival cells across the limbal barrier which enters the cornea and replaces corneal epithelial cells and disrupts

the balance, leading to angiogenesis. Hence, targeting miR-204-5p represents an interesting novel hypothesis of the pathogenesis of corneal neovascularization in AAK for future studies. In addition, these alterations observed were dependent on mutations in the *PAX6* gene. Thereby, this study highlighted the role of conjunctiva apart from the well-known abnormalities in limbal stem cells and corneal epithelial cell function in aniridia.

Hence, the deregulation of miRNAs in disease conditions can be harnessed as potential therapeutics by either miRNA replacement therapy using miRNA mimics or inhibition of miRNA function by antimiRs. Encouragingly, several miR-related clinical trials are ongoing for nonocular conditions such as miravirsen (for HCV infection), MRX-346 (for different types of cancers), MGN-4220 (for cardiac fibrosis), and many more. With the advancement in knowledge, emerging miRNA therapeutics will likely yield exciting breakthroughs in the current treatment options for ocular diseases. Besides therapeutic options, differentially expressed miRNAs can also be exploited as biomarkers for potential disease detection, for which a deeper understanding of the molecular mechanisms is needed.

Conclusion

Congenital aniridia is a complex disease affecting most structures in the eye and other organs. Based on the current understanding of aniridia, the significant challenges faced in the field are in its diagnostics and treatment. With a combination of newer technologies of next-generation sequencing (NGS) and whole-genome sequencing (WGS), the drawbacks of conventional technology platforms can be overcome. In addition, despite advancements in medical and surgical interventions, the long-standing problem of no long-term treatment options can be answered by introducing newer technologies such as gene therapy, gene silencing, and miRNA-based targeted approaches. Thus, exploring new generation modalities will hopefully improve the prognosis of this complex and devasting disease. The research in these areas continues to advance, offering hope to children affected by this condition.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient has given her/his consent for her/his images and other clinical information to be reported in the journal. The patient understands that her/his name and initials will not be published and due efforts will be made to conceal identity, but anonymity cannot be guaranteed.

Data availability statement

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Acknowledgments

The authors also wish to acknowledge Monica Chauhan's help in retrieving photographs and collecting the data.

Financial support and sponsorship

Nil.

Conflicts of interest

The authors declare that there are no conflicts of interests of this paper

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