Review

Guidelines for genetic study of aniridia

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A B S T R A C T

Introduction: Aniridia is a panocular disorder which occurs in 1/50,000 to 1/100,000 live births and can appear either in isolated form or in the context of a syndrome. Isolated aniridia is inherited as an autosomal dominant condition and is caused by mutations of the PAX6 gene. A variety of techniques and methodologies within molecular genetics and cytogenetics are used to study these mutations.

Objective: To identify the different aspects of this disease and to provide a guide for proper genetic diagnosis leading to improved clinical management of the disease.

Development: Aniridia is an autosomal dominant disease that primarily affects the iris, though it can impact most of the ocular structures. The disease is mainly caused by mutations in the PAX6 gene located on chromosome 11p13 which encodes a transcription factor that is involved in the development of the eye. Genetic analysis of aniridia is complex and requires the use of both molecular genetics and cytogenetics techniques. These procedures are indicated in all cases of aniridia. It is important to bear certain clinical and technical aspects in mind prior to starting analysis or providing genetic counseling for patients and their families.

Conclusions: The use of molecular genetic techniques in the genetic diagnosis of aniridia enables patients and their families to receive better clinical management.

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Guía para el estudio genético de la aniridia

R E S U M E N

Introducción: La aniridia es una enfermedad panocular con una incidencia de entre 1/50,000 a 1/100,000 nacidos vivos, que puede presentarse de forma aislada o en el contexto de un síndrome. Presenta una herencia autosómica dominante y en la mayoría de los casos está causada por mutaciones en el gen PAX6, para cuyo estudio de mutaciones se emplea una gran variedad de técnicas y metodologías de genética molecular y citogenéticas.

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Introduction

Aniridia (MIM: 106210) is a panocular disease with an incidence of between 1/50,000 and 1/100,000 live births. It can express on its own (mainly affecting the iris) or in the context of a syndrome (together with other systemic and/or ophthalmological alterations). In the majority of cases, aniridia is caused by mutations in the PAX6 gene (MIM: 607108), which encodes for a protein which is essential in the morphogenesis of the eye. Aniridia can be sporadic or familial and, with rare exceptions, of autosomal dominant inheritance.

There are many types of mutations responsible for aniridia. Among them we can find unique mutations (which are described below), chromosome microdeletions and deletions. Accordingly, for the genetic analysis of aniridia a large range of techniques and methodologies are applied, including molecular genetics techniques (direct sequencing, multiplex ligation probe amplification [MLPA] or compared genomic hybridization array [CGH-array]4 or ligand analysis5) as well as cytogenetic techniques (karyotype6 and on-site fluorescent hybridization [FISH]7).

In order to achieve adequate diagnostic and provide genetic counseling and efficient clinical management of this disease it is recommendable to follow the recommendations in this review, taking in all cases into account the criteria and professional experience of the physicians involved in the patient’s care and considering the specific clinical circumstances of each case.

Objectives

To collect the clinical and scientific aspects of the disease as well as the genetic aspects and the diagnostic methodology thereof in order to facilitate adequate genetic diagnostic and clinical management of patients and families affected by aniridia, in addition to providing a guide for the genetic diagnostic of this disease.

Development

Aniridia

Aniridia mainly involves the iris although this involvement can range from hypoplasia, lengthening and uneven surface mimicking coloboma, up to complete absence of structures. In addition, the involvement of the anterior eye chamber, retina, macula and optic nerve is common. Anterior segment alterations include keratopathy due to limbar dysfunction, dry eye, glaucoma (present in 50% of patients), cataracts, a lens subluxation and various chamber angle anomalies. Posterior segment alterations include macula and optic nerve hypoplasia. In addition, aniridia patients frequently exhibit strabismus and nystagmus which reduces the vigil prognosis.

Aniridia can appear in one eye or both, in isolation or associated to other alterations in syndrome cases such as:

- Rieger syndrome type I8 [MIM: 180500] which presents malformation of the anterior segment of the eye. 50% of cases exhibit blindness due to glaucoma9 and May associate systemic alterations.10
- Gillespie syndrome [MIM: 206700] which comprises aniridia, ataxia and mental retardation.11
- Peters syndrome [MIM: 604229] which associates anterior chamber malformations such as corneal opacity.12
- WAGR syndrome [MIM: 194072], the acronym being the names of the alterations comprised therein13 (Wilms tumor, Aniridia, Genital abnormalities and Retardation).

Genetic aspects

Inheritance pattern

Classic aniridia exhibits a dominant autosomic Mendelian inheritance pattern with a 50% risk of transmission to descendants.
The Gillespie syndrome with the dominant autosomic inheritance is also caused by mutations in the PAX6 gene (11p13).

The WAGR syndrome is sporadic and is caused by deletions in the 11p13 chromosomal region which includes, among others, the PAX6 and WT1 genes.

The Peters syndrome can be caused by mutations in PAX6 (11p13), PITX2 (4q25), CYP1B1 (2p22.2) and FOXC1 (6p25) genes. This syndrome is of recessive autosomic inheritance although dominant autosomic inheritance cases have been described.

The Rieger type I syndrome is caused by mutations in PITX2 (4q25) and FOXC1 (6p25) and is of dominant autosomic inheritance.

**Genetic defects in aniridia**

Classic aniridia is caused by mutations in the coding sequence or regulating region of the protein 6 gene located in the 11p13 chromosomal region. This gene comprises 13 exons and 22.4 kb and codes for a transcription regulating protein considered to be a master protein for the development of the eye.\(^{14,15}\) In the fetus, it is involved in the morphogenesis of the brain,\(^{16-19}\) the spinal chord,\(^{20}\) the olfactory epithelium\(^ {21,22}\) and the cerebellum.\(^ {23,24}\) In addition, it is essential for differentiating the alpha-pancreatic islets.\(^ {25}\)

The mutation of one of the 2 copies of PAX6 (mutation in heterozygosis) is enough to cause malformation in the majority of cases as this is the high penetration disease produced by haploinsufficiency.

The mutation associated to aniridia in heterozygosis is inherited from one of the progenitors in about two thirds of cases, with the other third being sporadic cases produced by \textit{de novo} mutations in the patient, even though said patient can in turn transmit the disease to his or her descendants.

The majority of mutations affecting only the PAX6 gene appear to cause functional loss\(^ {28}\) and are generally chromosome microdeletions or deletions which produce a stop codon (nonsense) or splice mutations or which alter the reading pattern (frameshift) which gives rise to truncated or nonfunctional proteins. Said mutations usually produce the aniridia phenotype, associated or not to extraocular alterations.\(^ {28}\)

Medical literature has reported the existence of 4 mutation hotspots in 4 CpG dinucleotides located in exons 8, 9, 10 and 11.\(^ {29}\) The transition-type mutations in these points account for about half of all the found nonsense mutations.

The mutations which truncate the protein are distributed throughout the PAX6 coding region, except in the 3’ half of exon 12 and in the exon 13 coding region.\(^ {29}\)

Approximately 10% of mutations in PAX6 involve the change of one amino acid by another one (missense). These mutations generally produce a less severe phenotype than aniridia\(^ {24}\) although this rule is not always fulfilled.\(^ {30}\)

Among the \textit{de novo} cases, one third exhibit the WAGR syndrome as the result of submicroscopic deletions in heterozygosis in chromosome 11 (11p13) which affect not only the PAX6 gene but also WT1\(^ {31}\) and other genes by an adjacent gene deletion syndrome.\(^ {32}\)

However, the existence of PAX6 regulating elements up to 200 kb beyond its encoding sequence must be taken into account, together with intra-gene elements. This explains the existence of chromosome restructuring cases\(^ {33}\) and mutations which do not alter the gene sequence (which remains intact) but affect the PAX6 control elements and produce the phenotype. This should be taken into account because it would explain the apparent absence of mutations in some familiar aniridia cases.\(^ {34}\)

**Phenotypes associated to PAX6**

PAX6 presents 14 exons that through alternate splicing give rise to 2 proteic isofoms, one of 422 aminoacids (PAX6) and another of 436 aminoacids (PAX6 5a).\(^ {35}\) Both are necessary for the development of the eye. In flies, gene PAX6 5a induces proliferation and PAX6 induces differentiation.\(^ {36}\) In vertebrates, during embryonic development PAX6 regulates the proliferation and differentiation of optic vesicle neuroepithelium progenitor cells.\(^ {37}\) In rats PAX6 participates in controlling corneal epithelium proliferation\(^ {38}\) and in maintaining the retinal progenitor cells of the ciliary body.\(^ {39}\)

Said gene expresses in various ocular structures, including the cornea, the lens the chamber angle, the ciliary body and all retina layers\(^ {38,39}\) and induces the differentiation of the lens and the retina.\(^ {40,41}\)

For the normal development of ocular structures the presence of PAX6 is necessary and in addition it should be in sufficient concentration.\(^ {42}\)

Mutations in the analog Pax6 gene lead to the eyeless phenotype in Drosophila\(^ {43}\) while in rats it can produce either small eyes or eyeless phenotype.\(^ {43,44}\)

The phenotype which is most associated to PAX6 mutations in humans is aniridia (panocular phenotype) and less frequently Peters anomaly\(^ {12}\) (MIM: 604229) (corneal central leukoma, absence of corneal posterior stroma and Descemet’s membrane as well as a variable degrees of iris and lens adherence to the central portion of the posterior cornea).

There are other phenotypes which are significantly less frequent than the 2 mentioned above such as ectopia pupillae\(^ {28}\) (MIM: 129750), foveal hypoplasia, in isolation\(^ {46}\) or associated to presenile cataracts\(^ {28}\) (MIM: 136520) and dominant autosomic keratopathy related to aniridia\(^ {46}\) (MIM: 148190). Genotypes which are very rarely associated to mutations in PAX6 are ocular coloboma\(^ {47}\) (MIM: 120200), optic nerve coloboma\(^ {47}\) (MIM: 120430) or bilateral optic nerve hypoplasia\(^ {47}\) (MIM: 165550). Some of the extracocular expressions associated to mutations in said gene include cranial or SNC malformations,\(^ {48}\) hypo- or anosmia\(^ {48}\) and glucose intolerance associated to aniridia.\(^ {49}\)

In aniridia cases (variations in the clinical expression of aniridia) a strict correlation between genotypes and phenotype has not been established. It is known that the clinical variations are very high because one mutation can presents different phenotypes\(^ {28}\) and intra-family variability has also been described. However, in general terms, there seems to be a relationship between the type of alteration induced in the protein and the ocular phenotype. Thus, the mutations that introduce premature termination codon are mainly associated to aniridia, whereas missense mutations are generally associated to non-aniridia phenotypes.\(^ {29}\)
**Recommendations for genetic study of aniridia**

**Prior recommendations**

When carrying out a genetics study with diagnostic purposes a number of factors must be taken into account prior to the genetic diagnostic (Fig. 1).

- Genetic counseling prior to sample taking. The patient must be informed about the clinical and hereditary implications of the genetic study to be undertaken. Genetic counseling is required by law (Art. 55 of Biomedical Research Act 14/2007):
  - Sample taking consists in 7 ml of blood in EDTA. For genetic studies blood samples need not be taken before breakfast.
  - The patient must be informed that a genetic study will be carried out to determine the genetic alteration which caused the disease, the hereditary model of said pathological process, the possible findings that could be obtained and the personal, clinical, reproductive and familial implications of said results.
  - Application for the study made by a physician. In addition, it is recommendable for this application to include a brief description of the patient symptoms and a detailed genealogical tree in family cases. The clinical indication of the study must be clearly stated.
  - The execution of a genetic study for aniridia is initially based on the existence of clinical suspicion and it must include at least the following data: date of birth, results of ophthalmological studies and interventions, the presence or absence of disease associated to aniridia (ophthalmological [glaucoma and leukemia] and/or systemic [genitourinary malformations, ataxia and/or psychomotor retardation]), the presence or absence of ophthalmological complications derived from aniridia (keratopathy, dry eye, glaucoma, cataracts, lens subluxation and/or chamber angle anomalies).
  - The family tree must include at least parents and siblings, indicating which relatives are affected and which are not.
  - A specific informed consent for the study to be performed duly signed by the patient (if the patient is not of age, by the father or mother), Act 14/2007 on Biomedical Research (Official Gazette 159, pp. 28826–28848 dated July 4, 2007), Act 41/2002 dated November 14, on Regulation of Patient Autonomy and Rights and Obligations in Clinical Information and Documentation (Official Gazette Dated November 15, 2002) and Additional Protocol to the Convention on Human Rights and Biomedicine Concerning Genetic Testing for Health Purposes. 50 Said consent is facilitated by the laboratory that carries out the genetic study.
  - Correct identification of the sample, both in the application as well as in the tube in which the extraction is placed. The use of at least 2 identifiers is recommended (e.g., patient name and date of birth). In addition it is convenient for the laboratory to identify the sample by numbers or codes.

The objectives of the genetic diagnostic of aniridia are:

(a) Confirmation of the diagnostic if the genetic defect is identified (although said confirmation is not discarded when the mutation is not identified).

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**Fig. 1 – Flow diagram of the genetic diagnostic of aniridia.**
(b) Evaluating the risk of Wilms tumor in congenital de novo aniridia in newborn or infant patients.

(c) Providing genetic counseling, i.e., to determine and report the risk of repetition for patients and their descendants, for healthy parents with affected children and for relatives (although this can also be predicted on the basis of a clinical diagnostic and the patient family tree even before executing the molecular study).

(d) Determine the evolution and prognosis of mutations with reported genotype–phenotype correlation.

(e) Carrying out a prenatal genetic diagnostic, not invasive as well as invasive.

The noninvasive prenatal diagnostic (NIPD) by means of fetal DNA study in maternal blood is feasible in:

- De novo cases. In a subsequent pregnancy the presence or absence of the mutation in the affected features of healthy partner is determined (where none of the partners carries the mutation of the affected fetus) in the blood of the pregnant woman.
- When the affected partner is the father (either due to de novo or familial mutations), the presence or absence of the father's mutation is determined in the blood of the pregnant woman.
- NIPD is feasible both in de novo as well as inherited cases, regardless of the sex of the affected individual.
- In the future, genetic treatment perhaps?

At present there is no treatment for curing aniridia. Existing treatments are focused on managing the alterations associated to this disease, including optometric aids for improving the visual acuity, drugs for keratopathy (serum rich in epithelial growth factors) and glaucoma, cataract surgery and glaucoma (trabeculectomy), intraocular lens implant (Morcher lens) for reducing photophobia, corneal and amniotic membrane transplant and in some cases esthetic treatment with artificial iris implant.

There are several lines of research for treating aniridia such as the transplant of autologous stem cells for treating limbar insufficiency and reconstructing the ocular surface or the use of polarized spectacles similar to an artificial functional pupil.

In recent years significant progress has been made in gene therapy in the context of ophthalmological diseases such as Leber congenital amaurosis. There is a broad range of therapeutic strategies based on genetics, including the “substitution” of the anomalous gene by a functional gene, “substitution” of the aberrant RNA, blocking the abnormal RNA by means of interference RNA, exon skipping technique or administration of the needed protein/enzyme. All the above strategies should be adapted to the environment of aniridia and assessed by means of clinical trials before including them in clinical practice.

**Technical considerations for the genetics study of aniridia**

There is a broad range of techniques and methodologies to detect mutations in the PAX6 gene. The choice of the most adequate methodology depends on several conditions which are intrinsic to each laboratory such as the availability of resources, experience or specific skills. In any case the selected technique/s should allow the identification of mutations and it is up to each laboratory to empirically determine the detection limits (sensitivity and specificity) of each technique as well as carrying out adequate controls (Table 1).

Various methodologies utilized for the genetic diagnosis of aniridia are listed below.

**Detection of unique mutations**

Screening techniques (denaturing High Performance Liquid Chromatography [dHPLC], High Resolution Melting [HRM], Single-Stranded Conformational Polymorphism [SSCP] and sequencing. These techniques are based on amplification by means of CRP of gene exons and adjacent sequences with subsequent electrophoresis to determine changes in sequence. The fragments with these changes are subsequently sequenced in order to adequately identify the defect. These techniques provide variable sensitivity (between 19% and 99%), depending on the method for existing defects in encoding sequence but not in regulatory elements.

**Detection of deletions (small and large)**

MLPA, FISH, CGH-array and family study with region markers are utilized. MLPA detects large duplications or deletions affecting exons of PAX6. There is a commercial MLPA kit (Salsa P219-B1) (MRC-Holland bv; Amsterdam, The Netherlands) which includes probes for each of the PAX6 exons excepting exon 6. Some commercial kits also include the WT1 region and are therefore very useful in de novo cases or where Wilms tumor is suspected. In general they are not useful for detecting unique mutations.

Cytogenetic techniques (high resolution karyotype, FISH) are useful for diagnosing a large chromosome restructuring of region 11p13 and which therefore could affect the PAX6 gene or its regulatory regions. These techniques are useful in WAGR syndrome in de novo aniridia cases and/or when alterations are not detected with the previously mentioned techniques. Cytogenetic techniques are also significant for genetic counseling as they could identify the risk of repetition in subsequent pregnancies.

As mutations could also exist beyond the encoding sequencing, it must be emphasized that the analysis of the PAX6 coding region is also able to detect mutations between 45 and 55% of familiar aniridia cases.

**CGH-array**

CGH arrays can be designed to detect variations in the number of DNA copies by means of compared genomic hybridization for studying region 11p13 genes (PAX6, WT1 and others). This technique enables the study of several genes at the same time as well as the encoding regions analysis and the PAX6 regulation region.

**Genetic ligand analysis**

In some circumstances and for diagnostic support purposes it could be useful to apply indirect analysis consisting in the study of microsatellite markers close to the PAX gene. The main limitations of the genetic ligand analysis is the...
Table 1 – Detection rate of mutations in PAX6 gene in different populations.

<table>
<thead>
<tr>
<th>Mutation cases</th>
<th>Clinical data</th>
<th>Technique</th>
<th>Population</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>5/9 family cases (56%)</td>
<td>Aniridia</td>
<td>SSCP</td>
<td>India</td>
<td>Neethirajan et al. (2006)</td>
</tr>
<tr>
<td>5/11 family cases (46%)</td>
<td>Aniridia</td>
<td>Sequencing</td>
<td>China</td>
<td>Wang et al. (2006)</td>
</tr>
<tr>
<td>30/54 cases (56%)</td>
<td>Aniridia</td>
<td>DGGE (ex 4-13) + SSCP</td>
<td>Europe</td>
<td>Vincent et al. (2003)</td>
</tr>
<tr>
<td>34/125 (27%)/33/37 (95%)</td>
<td>Aniridia/WAGR</td>
<td>Karyotype Sequencing</td>
<td>USA</td>
<td>Robinson et al. (2008)</td>
</tr>
</tbody>
</table>

* 13/18 family cases; 17/36 sporadic cases.

need of unequivocal clinical diagnostic of the disease and the participation in the study of several family members. An additional limitation is that the results will depend on the probability which varies according to the information provided by the markers in a specific family and the existence of possible recombination events.

In the case of prenatal diagnostic, the study of microsatellite markers segregation in the family (father, mother and fetus) must be included as diagnostic support and to discard possible maternal contamination of the fetal sample.

Conclusions

At present, the development of molecular genetic techniques and their application to the genetic diagnostic of aniridia enable improvements in the clinical management of patient and their relatives.

Conflict of interests

No conflict of interest has been declared by the authors.

REFERENCES


