1. Introduction

Aniridia (OMIM n° 106210) is an ophthalmologic disease characterized by several eye defects including iris absence or malformation, foveal hypoplasia, nystagmus. Affected subjects may also develop pre-senile cataracts and keratopathy. Moreover, they are at high risk for developing glaucoma. In addition to eye defects, patients may suffer of other sensory defects including reduced olfaction and hearing loss [1]. Aniridia is an autosomal dominant disease caused by loss-of-function mutations of PAX6 gene with virtually complete penetrance but variable expressivity [2]. PAX6 gene is located in the 11p13 chromosomal region and encodes a very conserved along phylogeny transcription factor, which is part of the PAX protein family [3]. In terms of causative genes, aniridia is very homogeneous: in only few subjects mutations located in genes different from PAX6 have been reported [2]. In a fraction of patients, the PAX6 gene defect consists in relative large deletions that cannot be identified by Sanger sequencing. When these deletions involve the WT1 gene, a more complex disease, named WAGR syndrome (Wilm’s tumor, Aniridia, Genitourinary anomalies, and Retardation) arises [4]. Aniridia-causing deletions, not detected by Sanger sequencing, may also be located outside the PAX6 encoding region, affecting sequences involved in the transcriptional control of this gene [5]. Altogether, chromosomal imbalances located in the 11p13 region are present in 20–30% patients with aniridia or WAGR syndrome.

Generally, chromosomal defects in the PAX6 gene region are detected by FISH or MLPA [6,7]. However, the FISH procedure requires cultures of cell patients and specific probes for each sub-region subjected to analysis. Moreover, The FISH is able to detect aberrations with a minimal length of at least several kbs [8]. On the other hand, MLPA is able to detect chromosomal aberration only in sequences covered by probes [9].

The comparative genomic hybridization array (CGHa) is the only microarray-based technology that is routinely used to detect chromosomal imbalances [10]. It can have a very high resolution power and it does not require specific probes for sub-regions subjected to analysis. In order to develop a simple and efficient procedure to detect PAX6 gene structural abnormalities, we have
set up a CGHa-based assay, focused on the 11p13 chromosomal region.

2. Materials and methods

2.1. Patients and DNA extraction

This study was conducted in agreement with the Declaration of Helsinki and was approved by the independent research ethics committee. Written informed consent was obtained from patients before their participation in this study. Thirty-five subjects were investigated by CGHa. Genomic DNA of patients was isolated from EDTA peripheral blood samples by using QIAamp DNA Blood Midi Kit according to the manufacturer protocol (Qiagen, Hilden, Germany).

2.2. Sanger sequencing and MLPA

In order to characterize subjects to be analyzed through CGHa, screening for PAX6 gene mutations was performed by Sanger sequencing and MLPA. To perform Sanger sequencing of the PAX6 gene, all coding exons and the corresponding intron/exon boundaries of gene was amplified by PCR with a specific subset of primers [6]. Sequencing reactions were analyzed by a 3730 DNA sequencer (Life Technologies). The nucleotide position of variants present in the coding regions refers to the cDNA sequence (NM_000280.4).

To perform MLPA analysis the SALSA MLPA reagent kit with probe mix P219-B2 developed by MRC-Holland (MRC-Holland, Amsterdam, Netherlands), was used. The kit contains probes to evaluate the presence of deletions and duplications in the 11p13 genomic region, which includes the following genes: PAX6 (15 probes), WT1 (5 probes), BDNF, FSHB, DCCDC1, RCN1 (2 probes each), ELP4, HIPK3, LMO2, EHF, CD44 (1 probe each) as well as in the 3q26 region, containing the SOX2 genes (3 probes). All procedures and data analysis were performed as indicated by the manufacturer, using a Mastercycler EP gradient thermal cycler (Eppendorf) for PCR amplifications and 3730 DNA sequencer (Life Technologies) for fragment analysis.

2.3. CGH array

CGHa was performed using a custom microarray kit (SurePrint G3 Custom CGH Array 4X180k, Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions. This is an oligonucleotide-based platform that allows high resolution molecular profiling of genomic aberrations in the 11p13 chromosomal region and, in particular, inside and around the PAX6 gene. According to hg19, the PAX6 gene is located between Mb 31,806 and 31,839. Thus, the average probe spacing was:

- from 30 Mb to 33 Mb average spacing = 100 bp
- from 20 Mb to 30 Mb and from 33 Mb to 43 Mb = 500 bp
- from 1 Mb to 20 Mb and from 43 Mb to 135 Mb = backbone

Labeling and hybridization were performed following the protocols provided by the manufacturer. Briefly, 1000 ng of purified DNA from a patient and a control (male and female DNA, Coriell Institute, Camden, NJ, USA) were double-digested with Rsal and Alul for 2 h at 37 °C. After 20 min at 65 °C, DNA of each digested sample was labeled by random priming for 2 h using Cy5-dUTP for the patient DNA and Cy3-dUTP for the control DNA. Labeled products were column-purified and prepared according to the manufacturer’s protocol. After probe denaturation and pre-annealing with Human Cot-1 DNA (Invitrogen, Carlsbad, CA, USA), hybridization was performed at 67 °C with rotation for 24 h. After two washing steps, the arrays were analyzed with the Agilent Scanner G2505C and Feature Extraction software (v 10.7.1.1). A graphical overview was obtained using Genomic Workbench Lite Edition software (v 6.5.0.18). Genome built hg19 was used to analyze the results. Our custom microarray is available upon request.

3. Results

In terms of causative genes, aniridia is a very homogeneous disease. In fact, only in very few patients have been found mutations in genes different from PAX6 [2]. However, mutations affecting PAX6 gene can be located either in the coding part of the gene or in transcriptional control elements [5]. Thus, in order to propose an efficient procedure to find structural mutations (deletions or duplications), a microarray was generated to perform CGHa assays in the PAX6 gene region. To identify even very small deletions/duplications, in the 3 Mb region in which PAX6 gene is centered the average spacing between probes is 100 bp (Fig. 1, panel A). A detailed image of the analyzed region is shown in the Supplementary Fig. 1.

By using PAX6 gene sequencing and MLPA of the PAX6 region, we have so far analyzed 125 aniridia and 4 WAGR patients, respectively. Causative point mutations and deletions have been found in 69 patients. In the present study, 35 subjects were subjected to CGHa analysis: 4 normal controls, 4 aniridia patients bearing known PAX6 point mutations, 4 aniridia patients bearing PAX6-containing deletions (previously detected by MLPA), 3 WAGR patients, 6 aniridia patients in which deletion only at level of the ELP4 gene has been previously found by MLPA, 14 aniridia patients in which neither deletion nor PAX6 point mutation were previously identified. Representative CGHa profiles are shown in panel B of Fig. 1. As expected, no deletions were found in normal controls and aniridia patients bearing PAX6 point mutations. Conversely, PAX6-bearing deletions were found in WAGR patients and in aniridia subjects with already known PAX6-containing deletions. In all five patients in which deletion at ELP4 gene level have been previously identified by MLPA, a deletion was found by CGHa. Absence of deletions was found in all the 14 aniridia patients negative after PAX6 gene sequencing and MLPA. Positions of deletions founded by CGHa in aniridia and WAGR patients are shown in Fig. 2.

4. Discussion

In this work, we set up a CGHa procedure to evaluate the presence of deletions of the PAX6 region in subjects affected by aniridia. Our approach is undoubtedly able to identify patients harboring deletions including the WT1 gene and, therefore, at risk for kidney tumors.

A major finding of this investigation is the missed identification of causative mutation, in a significant fraction of aniridia patients, after PAX6 exons (and splicing junctions) Sanger sequencing, MLPA and CGHa. The inability to find the causative mutation by using these technical approaches could be due to two not-mutually exclusive reasons. The first is that, even if for a small fraction of patients, aniridia can result from mutations in other genes, such as FOXC1, PITX2, PITX3 [2]. Indeed, recently, a missense mutation of the TRIM44 gene (p.G155R), has been found in a familial form of aniridia [11]. This gene encodes for a repressor of PAX6 expression and the p.G155R mutation enhances such a repressive effect. Thus, aniridia could be caused by mutations in genes different from PAX6. The second reason is related to the presence of point mutations in regulatory regions of PAX6 gene, as already demonstrated [5]. This possibility suggest that aniridia patients negative for point mutations and deletions after common procedures could be screened for point mutations in PAX6 regulatory regions.
Fig. 1. CGH array of PAX6 region. Panel A: the analyzed region in which most relevant gene are indicate by gray boxes (not in scale). Black boxes inside the ELP4 gene indicate PAX6 enhancers (not in scale). Panel B: examples of CGH array output. Subjects 1, 2 and 3 present deletions, while subject 4 is a negative control. Numbers flanking outputs indicate beginning and ending of the deletions. Green, red and blue rectangles on the left indicate positions of ELP4, PAX6 and WT1, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Patients in which deletions have been observed. The common region present in all patients is indicated by the vertical gray bar.
Another important finding of our study is that CGHa does not seem to be more effective than MLPA in finding deletions in the PAX6 gene region. In fact, though CGHa is able to detect all deletions found by MLPA, all negative patients after MLPA (and sequencing) analysis were also negative in CGHa assays. However, CGHa has a major advantage versus MLPA: provides detailed information on the border of the deletion. Considering these borders, the minimal deleted region contains the E1/SIMO enhancer, highlighting the role of this element in the control of PAX6 expression in the eye. Since the CGHa give the information whether a deletion in the ELP4 gene region includes the E1/SIMO, our assay has a diagnostic value higher than that given by available MLPA assays. Our data further indicate that mutation/rearrangement of long-range transcriptional control element are causative for a significant fraction of monogenic human diseases [12–15].

Author disclosure statement

No competing financial interests exist.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mcp.2016.12.001.

References

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