Retina

Evaluation of Italian Patients with Leber Congenital Amaurosis due to AIPL1 Mutations Highlights the Potential Applicability of Gene Therapy

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PURPOSE. To evaluate the suitability of gene delivery-based approaches as potential treatment of Leber congenital amaurosis 4 (LCA4) due to *AIPL1* mutations.

METHODS. Genomic DNA from patients was analyzed using a microarray chip and direct sequencing. A detailed clinical evaluation including fundus autofluorescence (FAF) and optical coherence tomography (OCT) was performed in patients with *AIPL1* mutations. *Aipl1* null mice and porcine eyes were subretinally injected with adeno-associated viral (AAV) vectors harboring the human *AIPL1* coding sequence.

RESULTS. We identified 10 LCA4 patients with mutations in *AIPL1*. The p.W278X sequence variation was the one most frequently found. Clinical assessment revealed common features including diffuse retinal dystrophies and maculopathy. However, optical coherence tomography showed partially retained photoreceptors in extramacular regions at all ages. The fundus autofluorescence was elicitable at the posterior pole and absent in the fovea. AAV-mediated gene transfer in *Aipl1* −/− mice was associated with restoration of AIPL1 and βPDE expression in photoreceptors and protection from degeneration. Administration of a clinically relevant dose of AAV2/8-*AIPL1* to the preclinical large porcine retina resulted in high level of AIPL1 photoreceptor expression in the absence of toxicity.

CONCLUSIONS. Using advanced imaging diagnostics we showed that maculopathy is a main feature of LCA4. We identified retinal areas at the posterior pole with surviving photoreceptors present even in adult LCA4 patients, which could be the target of gene therapy. The possible use of gene therapy for LCA4 is additionally supported by the protection from photoreceptor degeneration observed in *Aipl 1* −/− mice and by the high levels of photoreceptor transduction in the absence of toxicity observed after AAV2/8 delivery to the large porcine retina. (*Invest Ophthalmol Vis Sci.* 2011;52:5618–5624) DOI: 10.1167/iovs.10-6543

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L eber congenital amaurosis (LCA) defines a group of clinically and genetically heterogeneous blinding childhood diseases, characterized by early onset photoreceptor cell degeneration.

LCA is often considered as the most severe form of inherited retinopathy, which occurs in approximately 1 in 80,000 births.[1] Studies in animal models of LCA and more recently in humans using AAV-RPE65 gene therapy delivered to the retina have demonstrated success in restoring vision.[2–6]

LCA is usually inherited as an autosomal recessive trait, although dominant inheritance has been reported.[7] To date, 15 different genes (*GUCY2D, RPE65, CRX, AIPL1, CRB1, RPGRIP1, RDH12, IMPDH1, TULP1, CEP290, LCA5, SPATA7, RD3, LRAT,* and *MERTK*) have been identified, which harbor mutations resulting in LCA and related early onset retinal degenerations,[8,9] accounting for approximately 70% of all cases.

Mutations in *AIPL1* are responsible for LCA4 and account for approximately 5.3% of LCA.[8] The p.W278X mutation in the *AIPL1* gene is the most common over different populations.[10–12]

The phenotype of LCA patients with *AIPL1* mutations has been described as severe in nature.[12] Clinical features include some form of pigmentary retinopathy ranging from mild mid-peripheral salt and pepper-like retinopathy to severe choryoretinopathy with maculopathy, and varying degrees of optic nerve pallor. The macular involvement ranges from mild foveal dystrophy to atrophy, which is more pronounced in patients older than 6 years of age. Patients show markedly decreased visual acuities, ranging from 20/600 to no light perception, severe visual fields loss, and extinguished electroretinographic analyses (ERGs). In addition, keratoconus associated with cataract may be present in some cases.

The *AIPL1* gene encodes the aryl hydrocarbon receptor interacting protein-like 1 protein,[10] which has been shown to act as a specialized chaperone for rod phosphodiesterase (PDE), although further functional properties of this protein have not been fully elucidated yet. AIPL1 expression has been detected in both rods and cones during retinal development while in the differentiated retina expression seems restricted to rod photoreceptors.[13,14] Although it has been hypothesized that AIPL1 expression in cones is restricted to the retinal developmental period, very recent findings have shown that AIPL1 is expressed in human adult cones, where it may function to preserve cone activity and survival.[15]

Successful *AIPL1* retinal gene transfer using adeno-associated viral (AAV) vectors has been achieved in two different *AIPL1* mouse models,[16,17] suggesting that this strategy can be potentially applied to LCA4 patients. Indeed the safety and efficacy of AAV-mediated retinal gene transfer has been recently demonstrated in LCA2 patients.[2–6,18] However, gene therapy of LCA4 may have the potential for success only if retinal photoreceptors, the cellular targets of retinal transfer, are preserved.

Thus, a detailed characterization of LCA4 retinal structure and function is required to understand the potential applicability of gene therapy.

The purposes of this study are to describe the clinical and molecular features of LCA4 patients selected from a large cohort of Italian LCA patients, to identify retinal areas amenable to gene therapy, to define the temporal window for therapeutic intervention, and to study the effects of *AIPL1* retinal gene transfer in both a murine model of LCA and in the porcine retina.

MATERIALS AND METHODS

Patient Selection and Phenotype Analysis

Two hundred sixty Italian patients with LCA were selected at Referral Centre of Hereditary Retinopathies of the Department of Ophthalmology of the Second University of Naples.

The diagnostic criteria for LCA have been previously described elsewhere.[19] Ophthalmologic examination included best corrected visual acuity by Snellen charts or Teller Acuity Cards, measurement of objective refractive error after cycloplegia, slit-lamp biomicroscopy, and dilated fundus examination. Bilateral full-field ERGs were recorded (EREV 2000 Electrophysiology system; Pisa, Italy), with ERG-jet contact lens electrodes after International Society for Clinical Electrophysiology of Vision (ISCEV) standard guidelines.[20] This methodology was applied in both adult and pediatric patients.

LCA patients with identified *AIPL1* mutations were selected for a detailed phenotype analysis at the Department of Ophthalmology of the Second University of Naples.

Genetic counseling assessed that most cases were simplex cases, no patients had consanguineous parents and only two patients had one sibling affected.

Because nystagmus complicates fundus autofluorescence (FAF) and optical coherence tomography (OCT) recordings, a detailed ophthalmic evaluation including these tests was obtained only in 7 out of 10 patients with *AIPL1* mutations.

Autofluorescence was recorded with a standard confocal scanning laser ophthalmoscope (Heidelberg Retina Angiograph, Heidelberg Engineering, Heidelberg, Germany). To amplify the autofluorescence signal, we aligned the best five images obtained using the software integrated in the instrument, and calculated a mean image.

Cross-sectional retinal reflectivity profiles were obtained with spectral domain optical coherence tomography (Zeiss Meditec Cyrrus, Dublin, CA). Subjects underwent OCT imaging with the 512 × 128 scan pattern where a 6 × 6-mm area on the retina is scanned with 128 horizontal lines, each consisting of 512 A-scans per line (total of 65,536 sampled points) within a scan time of 2.4 seconds. The precise location and orientation of each scan were determined using the OCT simultaneous view video images.

As all patients but one had a variable degree of nystagmus, scans with the foveal depression at the center of the image were occasionally difficult to obtain in both eyes.

Mutation Analysis

Blood samples were collected from 260 Italian LCA patients. All procedures were adherent to the tenets of the Declaration of Helsinki, were approved by the local Ethics Committee and were performed after written informed consent was obtained from the patients or legal guardians in the case of children.

Genomic DNA was extracted from blood samples using standard techniques.[21]

Molecular analysis was performed using an integrated strategy involving the use of a genotyping microarray based on arrayed primer extension (APEX) technology (available in the public domain at http://www.asperbio.com/), and direct sequencing, as previously described.[19] Microarray findings were always validated by direct sequencing. In all patients in which microarray analysis yielded only one heterozygous *AIPL1* mutation, the entire *AIPL1* gene was analyzed by direct sequencing to identify the second mutated allele.

Gene Therapy in Animal Models

Generation of Constructs, AAV Vector Production and Purification. The human *AIPL1* coding sequence was amplified from human retina cDNA. The PCR was performed (Fast Start High Fidelity PCR System; Roche, Milan, Italy) to insert a *Not*I and a *Hind*III site at the 5′ and 3′ ends, respectively. The PCR product was then digested with *Not*I and *Hind*III and cloned into pAAV2.1-CMV-*eGFP* and pAAV2.1-Rho-*eGFP* plasmids[22] by removing the EGFP coding sequence (*Not*I–*Hind*III). AAV2/8-CMV-*hAIPL1* and AAV2/8-Rho-*hAIPL1* vectors were produced by triple transfection, purified by CsCl₂ ultracentrifugation, and titered (in genome copies [GC]/mL) using a real-time PCR-based assay and a dot blot analysis, as previously described.[22] AAV vectors were produced (AAV TIGEM Vector Core, Naples, Italy).

Vector Administration in Mice and Porcine Retina. All the experiments concerning animals were conducted according to the rules approved by the Italian Institution for animal research.

Mice. Aipl1 knock-out mice were kindly provided by Michael A. Dyer (Department of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, TN). Experiments were approved by the Animal Care and Use Committees of the Italian Ministry of Health and were adherent to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For subretinal vector administration, mice were anesthetized with an intraperitoneal (IP) injection of avertin at 2 mL/100 g body wt (1.25% wt/vol; 2,2,2-tribromoethanol and 2.5% vol/vol; 2-methyl-2-butanol; Sigma-Aldrich, St. Louis, MO) and viral vectors were delivered via a transscleral transchoroidal approach as described.[23]

Pigs. The Large White (LW) pigs used in our study were registered as purebred in the LW Herd Book of the Italian National Pig Breeders' Association. Pigs were starved overnight leaving water ad libitum.

Anesthetic procedure for ERG recording and surgical administration was performed as described.[24]

Subretinal Vector Administration. The procedure started with a transconjunctival scleral tunnel incision via pars plana parallel to the corneoscleral 16 limbus at 3.5 mm. The angle insertion of 23-gauge stiletto blade (Alcon, Fort Worth, TX) was performed for every case to facilitate the efficiency of self-sealing. Subsequently the light fiber, attached to the vitrectomy unit (ACCURS vitrectomy machine; Alcon) and either 38-gauge (Alcon) or extendible 41-gauge subretinal injection needles (DORC; Zuidland, the Netherlands) were respectively inserted through the two conjunctival incisions and into the two scleral tunnels. Therefore illuminating the posterior pole with the light fiber and

without vitreous removal, the injection in the subretinal space in the nasal area was performed with a 1-mL syringe connected to the sub-retinal needles, slowly and under direct observation with a stereoscopic microscope. Animals underwent handy slit-lamp biomicroscopy and indirect ophthalmoscopy before and immediately after injection, within three days after surgery and at kill four weeks post vector administration.[24]

Immunofluorescence Staining. Frozen retinal sections were washed once with PBS and then fixed for 10 minutes in PBS containing 4% paraformaldehyde (PFA). After fixation, sections were washed and incubated 1 hour in PBS containing 0.1% Triton X-100. Blocking solution containing 5% normal goat serum (Sigma-Aldrich) was applied for 1 hour. Primary antibodies were diluted in PBS and incubated overnight (O.N.). The secondary antibody was incubated for 45 minutes. The primary antibodies used were: rabbit anti-hAIPL1 (1:700; kindly provided by Michael E. Cheetham, University College London, London, UK), rabbit anti PDE6 β (1:100; Abcam, Cambridge, UK). The secondary antibody was Alexa Fluor 594 anti-rabbit, 1:1000 (Molecular Probes, Invitrogen, Carlsbad, CA). Vectashield (Vector Laboratories, Burlingame, CA) was used to visualize nuclei. Sections were photographed microscopically (with either an Axioplan microscope; Zeiss, or a Leica Laser Confocal Microscope System, Wetzlar, Germany).

Histologic Analysis. The tissue was processed and analyzed as described previously.[25] In brief, animals were killed, and the eyes were enucleated after an intracardiac injection of 4% PFA in PBS. The eyes were fixed overnight (O.N.) in 4% PFA, and transferred in sucrose steps of increasing gradient: 1 hour in 10% sucrose in PBS, 4 hours in 20% sucrose in PBS, and O.N. in 30% sucrose in PBS. The eyes were frozen in compound (Optimal-Cutting Temperature Compound; O.C.T.; Fisher Scientific, Pittsburgh, PA). For each eye, 150 to 200 (mice) or 200 to 300 (pigs) serial sections (12-μm thick) were cut along the horizontal plane; the sections were progressively distributed on 10 slides so that each one represents the whole eye at different levels. The sections were stained with hematoxylin and eosin according to standard procedures. Retinal histology was analyzed by bright-field light microscopy. The number of nuclei in the outer nuclear layer (ONL) of each eye was counted.

Electroretinographic Analysis. *Mice.* Scotopic ERGs were obtained with light flashes of 0.0001, 0.1, 5, 10, and 20 cd s/m^2 and photopic ERGs were elicited by a single flash of 20.0 cd s/m^2 in the presence of a constant background illumination set at 50 cd/m^2.[25] A-wave and b-wave were measured as described previously.[25]

Pigs. Bilateral full-field ERGs were recorded by a computer-based system (EREV 2000) and corneal contact lens electrodes with a Ganz-field stimulator according to the ISCEV protocol.[20]

Statistics

Data are expressed as mean $\pm$ SEM as indicated and compared by two-tailed Student's *t*-tests, two-sample equal variance.

RESULTS

AIPL1 Mutation Spectrum in LCA4 Patients

Ten patients (age range 5 to 79 years, mean age 29.3 $\pm$ 21.6 years) from eight independent families of Italian origin carrying disease-causing mutations in the *AIPL1* gene were identified, as indicated in Table 1. The analysis was mostly based on the use of an LCA mutation-specific microarray chip (see Materials and Methods, Mutation Analysis section) and was carried out on a total of 260 Italian LCA patients. Sequence analysis of the entire AIPL1 gene in three patients (A188:1, A188, and ARRP76) in which the genotyping microarray led to the identification of only one *AIPL1* mutated allele, allowed us to identify the second *AIPL1* mutations. Of these, the frame shift mutation c.96_97insGTGATCTT (p.G31fs) represents a totally novel mutation while the second, namely the missense mutation

TABLE 1. Clinical and Molecular Data of LCA4 Patients

Family	Patient ID	AIPL1 Nucleotide Variations	AA Variations	Age (y)	Age at Diagnosis (mo)	Nystagmus	Visual Acuity, RE	Visual Acuity, LE	Refraction, RE	Refraction, LE	Keratoconus	Lens	Fundus	AF	OCT μm (RE/LE)
1	A139	c.834G->A c.98_99 insTGATCTTG	p.W278X/p.F35fsX36	5	3	Yes	20/2400	20/2400	sf +7.00	sf +6	No	Clear	Salt-and-pepper	NP	47/74
2	A188:1	c.IVS5–10_786 del/ c.96_97 insGTGATCTT	Splice mutation/ p.G31fs	9	2	Yes	LP	LP	sf +6	sf +6	No	Clear	RP with macular dystrophy	Detectable	NP
2	A188	c.IVS5–10_786 del/	Splice mutation/ p.G31fs	13	2	Yes	NLP	NLP	sf +8.50 cyl −1 α40°	sf +8 cyl −1 α140°	No	Clear	RP with macular dystrophy	Detectable	NP/119
3	A184	c.834G->A c.834G->A	p.W278X/p.W278X	10	1	Yes	20/2400	20/2400	sf +2.50	sf +2.50	No	Clear	Salt-and-pepper	NP	108/NP
4	A182	c.834G->A c.834G->A	p.W278X/p.W278X	19	7	Yes	20/600	20/2400	cyl +2 α100°	cyl +2.50 α85°	No	Clear	RP with macular dystrophy	NP	96/NP
5	A197	c.834G->A c.834G->A	p.W278X/p.W278X	3	76	Yes	20/1200	20/1200	NP	NP	RE, keratoconus; LE, keratoplasty	PSC	RP with macular coloboma	NP	59/104
6	A183:1	c.834G->A c.834G->A	p.W278X/p.W278X	37	3	Yes	LP	LP	NP	NP	RE, keratoconus; LE, keratoplasty	RP with macular dystrophy	NP	NP	
6	A183	c.834G->A c.834G->A	p.W278X/p.W278X	44	2	Yes	LP	LP	NP	NP	No	Clear	RP with macular coloboma	NP	NP
7	A61	c.834G->A c.809G->A	p.W278X/p.R270H	41	6	Yes	20/1200	20/1000	sf −1.75 cyl −6 α70°	NP	RE, keratoplasty; LE, keratoconus	PSC	RP with macular dystrophy	Detectable	101/111
8	ARRP76	c.834G-> c.364G->AA	p.W278X/p.G122R	79	NR	No	HM	HM	NP	NP	No	RE NC pseudophakia	RP with macular dystrophy	NP	NP

ERGs were extinguished in all patients. cyl, cylinder; HM, hand motion; LE, left eye; LP, light perception; NC, nuclear cataract; NLP, no light perception; NP, not performed; NR, not referred; PSC, posterior subcapsular cataract; RE, right eye; RP, retinitis pigmentosa; sf, sphere.

c.364G->A (p.G122R) has been recently reported as amino acid change,[26] although with a different nucleotide change.

The p.W278X mutation was the one most frequently found in our cohort of LCA patients with an allelic frequency of 65% (13 out of 20 *AIPL1* mutations in probands; see Table 1). This mutation was found in all families analyzed except for one (family 2) and was present in homozygosity in five families.

Clinical Characterization of LCA4 Patients

The clinical features of the selected LCA4 patients are reported in Table 1. The age at diagnosis ranged between 1 to 7 months of age, confirming that the disease is characterized by a very early onset of clinical manifestations.

The ophthalmologic evaluation showed the presence of nystagmus in all patients but one, who was the oldest of the series. Light gazing was reported in three patients; in one patient night blindness, and in another photophobia, while no additional symptoms were reported in the remaining five patients.

Visual acuity was severely decreased in all patients and ranged from no light perception (NLP) to 20/600. The four youngest patients, (age range: 5–13 years; mean age: 9.25 ± 2.86 years) had a visual acuity between NLP and 20/2400.

Refraction, which was available in six patients, showed hyperopia in four patients, and a moderately high astigmatism in the remaining two.

Keratoconus was identified in three adult patients (30%), ranging from 36 to 41 years of age, in association with posterior subcapsular cataract (PSC). Electroretinograms were extinguished in all patients. Fundus examination revealed a salt-and-pepper retinal dystrophy in two young patients (Table 1 and Fig. 1A) while in eight patients (80%) typical features of retinitis pigmentosa (RP) were observed including a mild retinal vessel attenuation, bone spicule pigmentation, nummular pigmentation, and pale optic disc, in combination with a variable degree of maculopathy (Table 1 and Figs. 1C and 1F), varying from a mild foveal atrophy to a macular coloboma.

The OCT analysis was performed in six patients, two with salt-and-pepper retinal dystrophy and four with RP lesions and a variable degree of maculopathy. A reduced macular thickness was detected in all patients analyzed (age range 5 to 41 years; Table 1). Retinal lamellar structures were partially retained, displaying three retinal layers, with preservation of the outer nuclear layer (ONL) and photoreceptor inner/outer segment juncture (PSJ). The PSJ was preserved in the extrafoveal area in three patients, A139 (Fig. 1B), A182, and A184, and in the foveal and extrafoveal areas in patient A188 (Fig. 1G), while it was not present in patients A61 (Fig. 1D) and A197.

The autofluorescence analysis was performed in three patients with wide age range (9 to 41 years) having RP lesions and macular dystrophy at the fundus examination (Table 1). In all patients we observed the presence of fundus autofluorescence at the posterior pole that moderately decreased along the vascular arcades and the midperiphery, while no autofluorescence was detected in the fovea, due to an atrophic lesion (Table 1 and Figs. 1E and 1H).

AAV-Mediated Retinal Gene Transfer in *Aipl1* −/− Mice

To determine whether gene therapy can be a feasible therapeutic option for LCA4 patients we performed both proof-of-
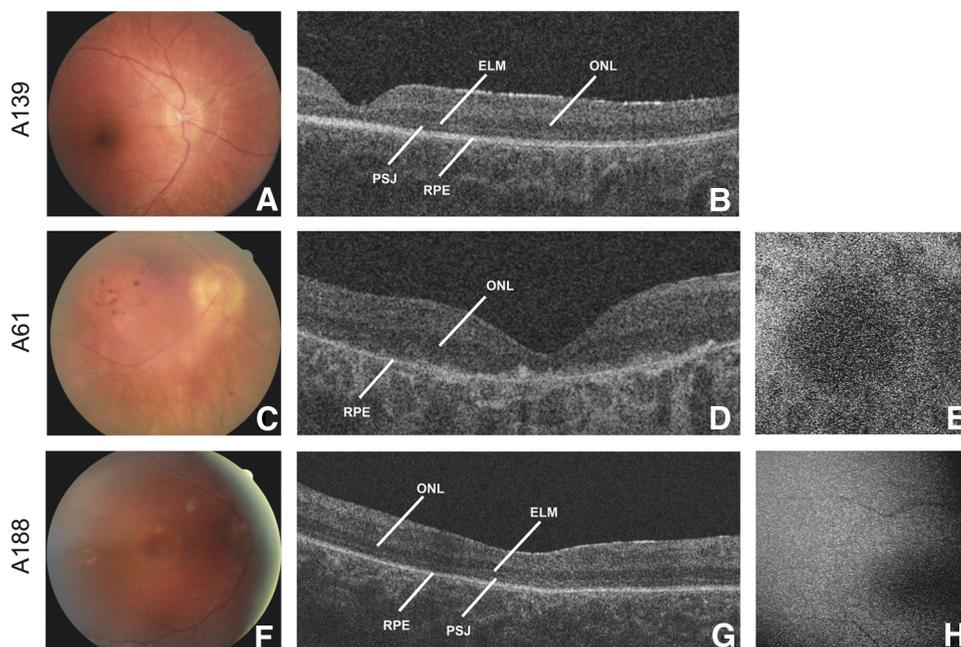

Figure 1. Fundus photographs, autofluorescence (FAF) and spectral domain OCT (SD-OCT) of LCA4 patients A139 at age 5, A61 at age 41, and A188 at age 13. Fundus image shows salt-and-pepper retinal dystrophy in patient A139 (**A**) macular atrophy and peripheral pigment migration in patient A61 (**C**), and foveal and parafoveal yellow discoloration in patient A188 (**F**). SD-OCT reveals retention of three retinal layers, with preservation of the outer nuclear layer (ONL) and the photoreceptor inner/outer segment (IS/OS) juncture in the extrafoveal area in patient A139 (**B**), multiple reflectance bodies visible in the residual ONL in patient A61 (**D**), and shows thinning of the photoreceptor layer in patient A188 (**G**). FAF shows the lack of autofluorescence in the foveal area along with more widespread peripheral areas of reduced signal in patient A61 (**E**), and demonstrates hypofluorescence at the fovea and low total autofluorescence in patient A188 (**H**). ELM, external limiting membrane; PSJ, photoreceptor inner/outer segment juncture; RPE, retinal pigment epithelium.

concept studies in *Aipl1*−/− mice and transduction efficiency experiments in a preclinical large animal model.

We first assessed whether AAV vector-mediated gene transfer can prevent retinal degeneration in *Aipl1*−/− mice, which shows a more severe and faster retinal degeneration than the patients studied here.[27–29] We generated two constructs containing the human *AIPL1* coding sequence (*hAIPL1*) driven by either the rhodopsin proximal promoter sequence (RHO)[30] or the cytomegalovirus promoter element (CMV).[30] Subretinal administration of AAV vectors harboring the RHO and CMV promoters results in both rod and cone transduction[30] with the RHO proximal promoter element restricting expression to photoreceptors.[30] We generated AAV vectors encoding *AIPL1* based on serotype 8 (AAV2/8-RHO-*hAIPL1* and AAV2/8-CMV-*hAIPL1*), which are currently considered the most efficient for photoreceptors transduction.[24,30] We injected *Aipl1* −/− mice subretinally with AAV vectors from postnatal day (P)4 to P8 before degeneration occurs.[27] To determine whether the treatment resulted in correct protein expression and function, we analyzed by immunofluorescence the expression of both AAV-delivered *AIPL1* and the endogenous murine βpde, which is a well-established target of *AIPL1* chaperone activity.[31] The expression pattern of both *AIPL1* and βpde resembled that observed in the human retina, which were analyzed in parallel (Figs. 2A–F). In particular, *AIPL1* expression was found in the photoreceptor inner segment (IS) while βpde in the outer segment (OS; Figs. 2A–F). In addition, histologic quantification of rows of photoreceptor nuclei in the outer nuclear layer (ONL) performed at P30 on retinal sections from *Aipl1* −/− mice showed preservation of photoreceptors in the retinas treated with AAV (*n* = 6 retinas either injected with AAV2/8-CMV or −RHO-AIPL1, mean ± SEM, 5 ± 1 rows of nuclei in each set of retinas analyzed) compared with contralateral untreated retinas (*n* = 4 untreated retinas, mean ± SEM, 1 ± 0.2 rows of nuclei, *P* treated versus untreated ≤ 0.001; Figs. 2A–F). Wild type, age-matched control mice present 11 rows of nuclei (*n* = 3 wild type, mean ± SEM, 11 ± 1.2) in the outer nuclear layer. Despite the preservation of retinal structure, electroretinographic analyses (ERG) responses were negligible in both CMV- and RHO-treated animals analyzed at P30.

We next sought to determine AAV2/8-mediated *AIPL1* transduction efficiency in large animals with a protocol potentially used for a human clinical study. To this end we have used a dose of AAV2/8 similar to that used in the RPE65 clinical trial conducted by Maguire et al.[5] In addition, we used a vitreoretinal surgical protocol for subretinal administration of the vector similar to that used in RPE65 patients.[2–6] We subretinally injected monolaterally 1 × 10^{10} (genome copies) of AAV2/8-CMV-*hAIPL1* vector (total volume of 100 μL) in 2 eyes in the avascular nasal cone-enriched area of the posterior pole of 2-week-old pure Large White (LW) female pigs (live weight, 30 ± 2 kg).[24] The 2 animals underwent ophthalmologic examination 3 days after surgery and at kill 4 weeks after vector administration. At both time points no signs of adverse events including inflammation or detachments were observed (data not shown).

To assess pattern and levels of AIPL1 expression we performed retinal confocal microscopy analyses of retinal sections from the injected area and, as negative controls, from both the noninjected areas of the same and of the contralateral uninjected eyes. Robust expression of human *AIPL1* (without cross-reaction between endogenous porcine *Aipl1* and vector derived *hAIPL1*; Fig. 2I) was observed in both the retinal pigment epithelium (RPE) and cone and rod photoreceptors outer segments, and to a lower extent in nuclei of the retinas treated with AAV2/8-CMV-*hAIPL1* (Figs. 2G and 2H).

In addition, histopathological analyses (hematoxylin and eosin staining, H&E) of retinal sections revealed normal retinal histology, including normal thickness of both outer and inner nuclear layers and lack of inflammatory cell infiltrates in the treated eyes (Figs. 2J and 2K).

In addition, to determine whether AAV vector administration, *AIPL1* expression and/or the surgical technique applied resulted in detrimental effects on retinal function, full-field ERGs were recorded at baseline and before kill. Both rod and cone isolated and combined responses of treated eyes showed no statistical differences compared with baseline measurements (baseline pretreatment *n* = 2, photopic, 129 μv; scotopic, 43 μv, and maximal response 178 μv; posttreatment *n* = 2, photopic, 135 μv; scotopic, 44 μv, and maximal 193 μv).

DISCUSSION

This study provides a comprehensive clinical description of a cohort of 10 LCA4 patients with *AIPL1* mutations. Molecular analysis revealed that the p.W278X nonsense mutation was the
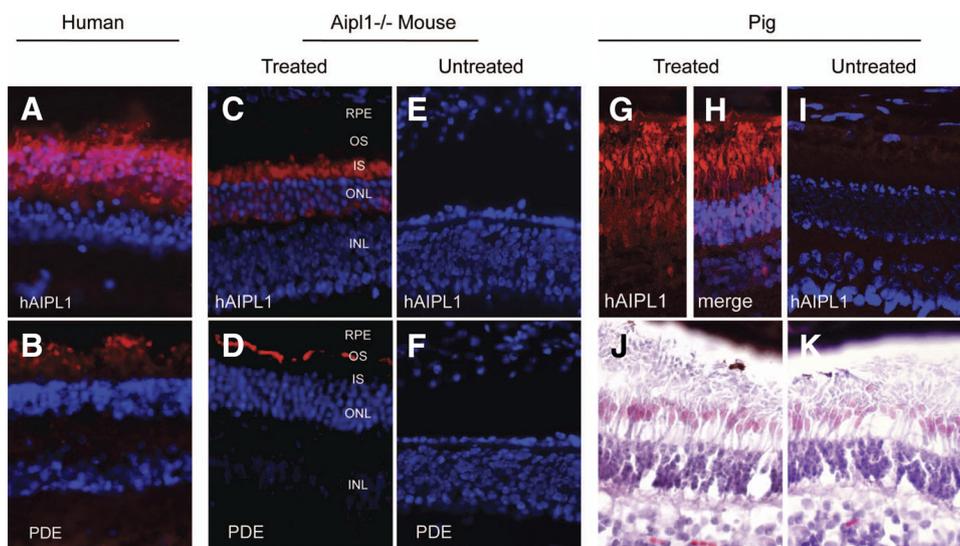

FIGURE 2. AIPL1 protein expression in human, murine, and porcine retinas. AIPL1 and βPDE immunofluorescence staining (*red*) of the human retina (**A**, **B**) and of AAV-treated and untreated murine *Aipl1*−/− (**C–F**), and porcine (**G–I**) retinas 3 and 4 weeks after injection, respectively. Subretinal injection of AAV2/8-RHO-hAIPL1 in murine *Aipl1*−/− resulted in preservation of outer nuclei from degeneration (**C**) expression in the photoreceptor inner segment (IS) and the correct translocation of βpde to the outer segment (OS; **D**). Confocal microscopy analysis shows that subretinal injections of AAV2/8-CMV-AIPL1 vector in porcine retina resulted in high levels of AIPL1 expression in rod and cone photoreceptors OS and to a lesser extent to outer nuclear layer (ONL; **G**, **H**). Hematoxylin and eosin (H&E; **J**, **K**) staining of retinal sections showed normal retinal histology without inflammatory infiltrates in treated compared with untreated retinas (**H** and **I**, respectively). INL, inner nuclear layer; RPE, retinal pigment epithelium.

most frequent allele identified, thus suggesting it is a founder allele in Italy similar to what has been previously described for other populations.[10,11]

The clinical analysis of our LCA4 patients, spanning over a wide age range, revealed that all patients presented with a severe early onset retinal dystrophy that was diagnosed as LCA.

All patients showed poor visual function, that was detected early in life in agreement with a previous report.[12] Hyperopia was the most common refraction defect, detected in approximately 66% of the patients analyzed.

In addition, subcapsular cataract, in combination with keratoconus, was detected in approximately one third of adult patients, in agreement with previous reports.[12]

The patients shared a common clinical retinal picture characterized by diffuse retinal dystrophy that frequently appeared as an RP-like lesion in combination with maculopathy and, less frequently, as a mild midperipheral salt-and-pepper dystrophy, in absence of clinical signs of maculopathy at the fundus examination. To this end, previous findings in a few patients have suggested that a maculopathy of variable appearance is present in most patients, even in the younger ones who show an abnormal indistinct foveal reflex, which likely represents an early stage of maculopathy.[12,32,33]

An OCT examination was performed in a subset of our patients. Independently of the age of the patients at the time of the analysis, we observed a variable decrease of retinal thickness in the macula, indicating that a variable degree of maculopathy is already present in childhood, even when signs of maculopathy are not detectable by ophthalmoscope (patients A139 and A184; Table 1). Considering that the youngest patient (A139, 5 years old) already displayed a thinned macula but preserved structures in the rest of the retina, one can argue that the maculopathy might also be due to a developmental defect leading to fovea hypoplasia rather than a degenerative defect. In this respect, the analysis of additional LCA4 infants is required.

Our results confirm that the presence of maculopathy is a shared clinical finding in LCA4 patients and could be considered as an additional diagnostic criterion that may suggest the presence of *AIPL1* mutations in LCA patients.

Despite the severity of the retinal phenotype, as detected by fundus and ERG examinations, we observed the presence of autofluorescence at the posterior pole in all 3 patients analyzed. This suggests that AIPL1 deficiency does not halt the formation of lipofuscin fluorophores. Fundus autofluorescence is an index of lipofuscin accumulation in the retinal pigment epithelium (RPE) and its preservation reflects the level of metabolic activity, thus indicating at least a partial presence of structurally intact photoreceptors and photoreceptor/RPE complex.[34,35] The presence of fundus autofluorescence at the posterior pole in two young patients (A188:1 and A188), showing severe visual impairment ranging from light perception (LP) to no light perception (NLP) and maculopathy, was notable and suggested a partial presence of surviving photoreceptor cells despite the severity of clinical manifestations. Furthermore, the similar findings in an older patient (A61), affected by retinal dystrophy for > 40 years, suggested that surviving dysfunctional photoreceptors exist even later in life.

The hypothesis of surviving photoreceptors was also supported by the OCT images that showed partially retained retinal lamellar structures, with the presence of three retinal layers, and a visible photoreceptor inner/outer segment juncture.

In our patient sample, characterized by a prevalence of the homozygous p.W278X *AIPL1* mutation, the severity of the retinal lesions appears to be less severe than that described recently by Jacobson et al.[26] Screening of increasingly large numbers of patients would help to determine whether this difference can be explained by genetic factors such as the nature of the causative mutations, the presence of modifier genes, or both.

Thus, based on FAF and OCT results we predict that retinal areas at the extrafoveal region could be amenable for gene therapy treatment and that the temporal window for successful treatment may not be as restricted as expected. In fact, it is extremely important to distinguish between cell death and cell dysfunction when considering therapy. If the photoreceptor cells are viable but dysfunctional, gene therapy may allow recovery of photoreceptor function. The contribution of rescued extrafoveal region to vision in these patients is difficult to predict: however, our previous experience in the LCA2 trial suggest that this may be relevant for visual function recovery. Indeed, in the LCA2 gene therapy trial we have observed significant improvement in visual function after extrafoveal vector administration.[4] Nonetheless, further studies will be necessary to determine the net impact on visual function recovery of AAV-AIPL1-mediated gene transfer to extrafoveal region of LCA4 patients.

Visual function depends not only on the amount of viable photoreceptors but also on visual experience and cortical development. The early severe vision loss in *AIPL1* patients determining stimulus deprivation amblyopia could be a complicating factor preventing gene therapy success. A possible strategy could be to treat pediatric patients because visual recovery from amblyopia is possible in early infancy.[36,37]

The analysis of the *Aipl1−/−* mouse retina shows that Aipl1 deficiency leads to a severe and rapidly progressive retinal degeneration, indicating that the temporal relationship between photoreceptor functional and structural impairment differs between humans and mice. Nonetheless, we showed that *AIPL1* gene delivery before degeneration provides the correct photoreceptor localization of the protein, which in turn stabilizes βpde protein expression and protects photoreceptors from degeneration. This is similar to what has been previously observed by Sun et al.[16] and Tan et al.[17] While they used the ubiquitous CMV and the photoreceptor-specific rhodopsin kinase promoters, we have used the CMV and, as photoreceptor-specific, the rhodopsin promoters. In addition, our data show that subretinal administration of a clinically-relevant dose of AAV2/8[2-6] enables robust and safe transduction of both rod and cone photoreceptors of the large pig retina. The absence of toxicity observed in pigs after retinal *AIPL1* gene delivery, suggests that AIPL1 overexpression in addition to the endogenous protein is not detrimental. Our data, together with those provided by Sun et al.[16] and Tan et al.,[17] and in combination with the partial preservation of photoreceptor structure we observed in LCA4 patients, support the use of gene therapy for this form of LCA.

Acknowledgments

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