Master of Science

Development of a novel humanized Knockin mouse of retinitis pigmentosa with IMPDH1 mutation

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Development of a novel humanized Knockin mouse of retinitis pigmentosa with IMPDH1 mutation

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Summary

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Purpose: Retinitis pigmentosa causes blindness from retinal damage and has no cure. To advance treatment options, animal models with retinal degeneration are necessary. IMPDH1 is a gene associated with the disease, and an animal model with a mutated IMPDH1 gene has been developed for further research.

Methods: Researchers used CRISPR-Cas9 to create a retinal degeneration model in mice with a human mutation. They replaced a nucleotide with ssODN and studied the mice's characteristics with imaging and tests until they were 7 months old.

Results: At 4 weeks, genotypes show a difference in retina thickness. Wild types maintain the layer until 7 months; however, it thins after 4 weeks. Homozygotes experience retinal detachment around 8 weeks and severe degeneration by 7 months. Heterozygotes have thinner retinas and slower pigmentation progression.

Conclusions: These results suggest that the phenotype appeared differently depending on the genotype (wild, heterozygote, and homozygote) of the produced IMPDH1 mutated humanized mouse. Accordingly, it was confirmed that the IMPDH1 gene is autosomal dominant, and the degree of symptoms varies depending on the genotype

Keywords: Retinitis pigmentosa, Autosomal dominant, IMPDH1 gene mutation, Humanized KI mouse, Retinal degeneration

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Introduction

Retinitis pigmentosa (RP) is a disease associated with gradual degeneration of the retina, in which patients have night blindness followed by reduced visibility, resulting in tunnel vision and eventually complete blindness. RP causes rod and cone cell apoptosis, ultimately decreasing the thickness of the outer retina's outer nuclear layer and causing lesions and retinal pigment deposits in the fundus [1]. RP is a group of inherited disorders affecting 1 in 3000-7000 people. RP can be inherited as an autosomal dominant (AD), autosomal recessive (AR) or X-linked recessive (XR). Autosomal Dominant RP is associated with 22 gene mutations [2,3].

Despite advancements in medical research [4], there is currently no definitive treatment for RP. Various approaches, such as gene therapy, retinal implants, and stem cell transplantation, have shown promise, however their effectiveness is limited. Developing effective treatment methods for RP requires an appropriate animal model to study the disease's progression, understand its underlying mechanisms, and test potential therapeutic interventions.

Animal models, including mice [5,6,7], rats [8], dogs [9,10], and pigs, have been widely used in RP research. These models often exhibit similar disease characteristics as humans, such as progressive degeneration of photoreceptor cells and visual impairment. Animal models provide a valuable platform for preclinical testing of potential therapies, allowing researchers to assess safety, dosage, and effectiveness before moving on to human clinical trials. They enable the evaluation of interventions such as gene therapy, stem cell transplantation, and drug therapies, providing important data on their potential benefits and limitations.

Among the AD genes that cause RP, IMPDH1 is believed to be one of the causative genes in previous studies [11], however, its pathological characteristics were unclear. IMPDH1 catalyzes the rate-limiting step in the de novo synthesis of guanine nucleotides, impacting GMP, GDP, and GTP cellular pools. Guanine nucleotide homeostasis is central to photoreceptor cells, where cGMP [12-14] is the signal-transducing molecule in the light response [15]. Mutations in IMPDH1 lead to inherited blindness [16].

This study aimed to develop an experimental mouse model by inserting the IMPDH1 mutation gene, which induces an AD pattern of RP in humans. We aimed to investigate the phenotypic characteristics of this animal model and assess its potential as a model for studying RP.

Materials and Methods

Animals

All experimental procedures followed the statement for using animals in ophthalmic and vision research provided by the Association for Research in Vision and Ophthalmology.

All experimental C57BL/6j mice (JA BIO, Gyeonggi-do, Korea) were housed in a specific pathogen-free animal facility at the Asan Institute for Life Sciences, in the Asan Medical Center. All animal care and experimental procedures were reviewed and approved by the Institution Animal Care and Use Committee (IACUC) of the Asan Institute for Life Sciences (approval number, 2021-12-339).

ssODN(single-base replacement using a single-stranded oligo DNA nucleotide) is injected into the mouse's fertilized egg to induce gRNA (guide RNA) that complementarily binds to the IMPDH1 gene site of the mouse corresponding to the site containing the (c.947G) of the human IMPDH1 gene and mutations corresponding to the human R316P mutation in the mouse genome. The fertilized egg injected with ssODN was cultured to form an embryo, then transplanted into a surrogate mother to obtain the mouse-containing reproductive cells in which the IMPDH1 gene in the genome contains a mutation corresponding to the human R316P mutation **(Fig. 1)**. The mouse obtained in this step is hybridized with a wild-type mouse to get the mouse in which the IMPDH1 gene in the genome contains a mutation corresponding to the human R316P mutation as a heterozygote.

All mice were anesthetized before the experiments by intraperitoneal administration of a mixture of alfaxalone (Alfaxan, 0.4 mL/kg; Jurox, New South Wales, Australia) and xylazine (Rompun, 0.6 mL/kg; Bayer Korea Ltd. Seoul, Korea).

Fundus photography and optical coherence tomography

Mouse was evaluated by fundus photography and optical coherence tomography (OCT) at 4, 8, 10, 16 weeks and 7 months of postnatal age. After anesthesia, the pupils were dilated with Mydrin-P (0.5 % tropicamide and 0.5 % phenylephrine hydrochloride; Santen Pharmaceuticals, Osaka, Japan) for imaging the retina. Hycell physiological solution (2 % hydroxypropylmethylcellulose; Samil

Pharmaceuticals, Korea) was applied to the cornea to prevent dryness. The refraction was equalized using a microscope coverslip. All the fundus and OCT images taken were stored, and the data were processed using Micron IV software (StreamPix; NorPix, Inc. Montreal, Quebec, Canada). Fundus and OCT imaging (MICON IV; Phoenix Research Labs, California, USA) were used to observe clinical signs of retinal degeneration [17, 18].

Electroretinogram

The mouse was dark-adapted overnight before the recording. General anesthesia was induced by intraperitoneal administration of an alfaxalone and xylazine mixture. Only dim red light was employed when measuring the scotopic and photopic reactions [18, 19]. All electroretinogram (ERG) measurements were obtained using a Phoenix Micron IV system (Phoenix Research Labs, California, USA) and processed with LabScribeERG software (ver. 3; Phoenix Research Labs). After applying Hycell physiological solution (2 % hydroxypropylmethylcellulose; Samil Pharmaceuticals, Korea) to mouse corneas, they were identified using a gold-plated objective lens. For measurement, reference needle electrodes were inserted in the forehead and ground electrodes in the skin at the tail. During electroretinogram recording, mice were on a heating pad to maintain an appropriate body temperature.

Rod-dominated responses to white light flash over a 1.3 to 4.6 (log cd/m^2) log unit range of intensities were recorded. Cone-dominated responses were obtained with white flashes over a 2.8 to 4.6 (log cd/m^2) log unit range of intensities after 10 minutes of light exposure for complete light adaptation. Signals were sampled every 1500 ms (scotopic) and 300 ms (photopic). For each stimulus condition, responses were computer averaged; up to 20 records were averaged for the weakest signals [20, 21]

Responses were recorded in IMPDH1 mutation KI mice (homozygote, heterozygote) at 8 weeks to 7 months. Comparisons with age-matched wild types were performed.

Histologic analysis

At 2 weeks and 8 months of postnatal age, eyes were enucleated after mice were killed in a CO2

chamber and perfused with 4 % paraformaldehyde (PFA). The eyes were fixed overnight with 4 % PFA, embedded in paraffin, and cut into 4 μm sections for hematoxylin and eosin (H&E) staining and immunofluorescence assay. Specimens were stained with H&E. A microscope (Olympus CX41; Olympus America, PA, USA) was used to obtain H&E data.

For immunofluorescence asay, the retinas were blocked with 5 % normal goat serum in PBST (0.5 % Triton X-100 in PBS) for 1 h and treated overnight with an anti-IMPDH1 antibody (22092-1-AP; Proteintech, 1:100 dilution) at 4 ℃. Following a wash in 0.5 % PBST, the samples were incubated for 2 h at room temperature with a secondary antibody (DI-1594, goat anti-rabbit IgG H&L DyLight 594, Vector Labs, 1:1000 dilution). The samples were washed two times in 0.5 % PBST and mounted with a mounting medium with DAPI (ab104139, Abcam, Cambridge, UK). DAPI dyes identified the nucleus. A Zeiss LSM 710 confocal microscope (Carl Zeiss, Berlin, Germany) was used to obtain immunofluorescence data.

Terminal deoxynucleotide transferase nick-end labeling (TUNEL) assay

Photoreceptor apoptosis was determined by using the terminal deoxynucleotide transferase nick-end labeling (TUNEL) assay with a DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA). For TUNEL, sections were deparaffinized, rehydrated, treated with Proteinase K, reacted with TdT/ nucleotide mix (containing fluorescein-12-dUTP), and counterstained with DAPI-blue. All specimens were examined on a Zeiss LSM 710 confocal microscopy system (Carl Zeiss Meditec AG, Jena, Germany). The TUNEL assay was compared between IMPDH1 mutation KI mouse (homozygote, heterozygote) and age matched wild type mouse (2 weeks, 7 weeks).

Genotyping and sequencing

Mice genotyping was conducted using genomic DNA extracted from tissues obtained from tail biopsies. For genotype analysis, PCR was performed using the primer pair (forward primer (5′-TCAGGGGTCTCTCGTTCCAT-3′) and reverse primer (5′-CAAAGTGGGGAGGGAGCAAA-3′)). After purifying the PCR product, sequencing was performed using the oligomer (5′-CCCTGCTTCTCTCTCCTCCT-3′) to confirm the production of the IMPDH1-R316P mutant founder

(F_0) mouse. As shown in **Fig. 2a**, it was confirmed that two IMPDH1-R316P mutant mice (#26 and #35) were generated. These IMPDH1-R316P mutant founder mice were hybridized with wild-type mice to confirm that the IMPDH1-R316P mutant allele identified in the mutant founder was germ-line transmitted to descendants (F_1) (**Fig. 2b**). Accordingly, an IMPDH1-R316P mutant mouse line was established.

Statistics

Excel (Microsoft, Washington, USA) and GraphPad Prism v. 8.0.1 (GraphPad Software, San Diego, CA, USA) were used for statistical analysis. Data are shown as the mean ± standard deviation (SD). The Shapiro–Wilk test was used to test for normal distribution. The student's t-test was used to compare normally distributed metrics. . P value <0.05 was considered statistically significant.

Results

Generation of humanized IMPDH1-R319P mutation KI mice

The mice model was generated using the CRISPR-Cas9 system using ssODN. The operation of the CRIPSR-Cas9 and C57BL/6NTac mouse fertilized eggs was carried out according to the results of previous studies conducted by the inventors [22], and ssODN was added to this. As shown in **Fig. 2a**, it was confirmed that two IMPDH1-R316P mutant mice were generated (F_0). These IMPDH1-R316P mutant founder mice were hybridized with wild-type mice to confirm that the IMPDH1-R316P mutant allele identified in the mutant founder was germ-line transmitted to descendants (F_1) (**Fig. 2b**). Accordingly, an IMPDH1-R316P mutant mouse line was established. The phenotype of this mouse was observed by fundus, OCT, ERG, and histologic analysis.

Clinical phenotype

No significant difference was observed between genotypes until 2 weeks old. However, at 4 weeks old, there is a clear difference in the thickness of the retina and the clarity of the layer. In the case of the wild-type, the layer is normally maintained until the age of 7 months, and the fundus imaging shows that the thickness of the retina is thinner at 4 weeks, and the optic nerve and blood vessels are quite pale. Retinal detachment was observed in homozygotes around 8 weeks. At the age of 7 months, it was confirmed that there were few layers of the retina left in the homozygote and that retinal degeneration progressed severely, resulting in mottling. Although a heterozygote was not as severe as a homozygote, the retina was thinner than the wild-type, and retinal pigmentation was progressing.

Comparison of patient phenotype with the IMPDH1-R319P mutation KI mouse

The produced IMPDH1-R316P mutant mouse animal model was compared and observed with the patient phenotype. For the patient who was diagnosed with Lever Congenital Amaurosis (LCA), genetic analysis confirmed that the c.947 G>C, p.Arg316Pro mutations showed heterozygote mutations. Overall retinal degeneration and atrophy were observed in the fundus and OCT images

(**Figs. 3a, 3b**). ERG (extinguished ERG) was observed, showing the same expression as IMPDH1-R316P heterozygote and homozygote mutants produced in this invention [3] (**Fig. 4**).

Functional analysis of the IMPDH1-R319P mutation KI mouse

We performed an ERG to evaluate visual function. At 8 weeks of postnatal age, the heterozygote has a certain reaction. However, the homozygote does not react and shows a flat graph. In the 7-month postnatal age, heterozygotes and homozygotes are unresponsive and show a flat graph. These ERG results indicate that IMPDH1-R319P mutations can contribute to visual impairment in mice (**Fig. 4**).

Histologic analysis

In H&E, at 2 weeks of postnatal age, the retinal layer appears well preserved in all genotypes. However, at 7 months of postnatal age, it was observed that the overall retinal thickness of heterozygote and homozygote mice decreased compared to WT mice. The outer nuclear layer (ONL) aggressively reduced among the 10 layers of the retina. In homozygotes, ONL seems to have disappeared completely.

In the immunofluorescence assay, it can be seen that the degree of expression of IMPDH1 varies from 2 weeks of postnatal age. Homozygote is rarely expressed compared to heterozygote and WT. At 7 weeks of postnatal age, the expression of IMPDH1 in heterozygotes was weakened compared to 2 weeks of postnatal age (**Fig. 5**).

TUNEL assay

In the TUNEL assay, there was a difference between genotypes. At 2 weeks of postnatal age, we observed that heterozygotes and homozygotes had much apoptosis, while WT tissue showed no signal. The signal was specific to ONL; there are few signals in other layers—much more in the homozygote than in the heterozygote, and no more signal at 7 months postnatal age (**Fig. 6**).

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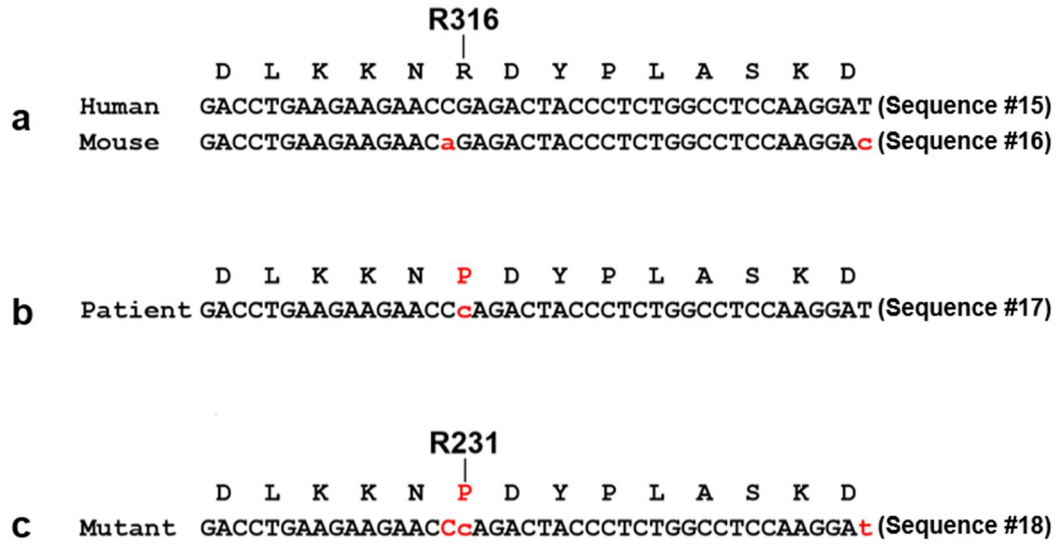


Figure 1. Design process for producing a humanized IMPDH1-R316P mouse model with a mutation (IMPDH1-R316P) in which R316 amino acids are changed to proline (P) in human IMPDH1 proteins. (a) Wild-type IMPDH1 gene. **(b)** Patient-derived mutation (IMPDH1 NM_000883.3: c.947G>C, p.Arg316Pro). **(c)** Humanized IMPDH1-R316 mouse.

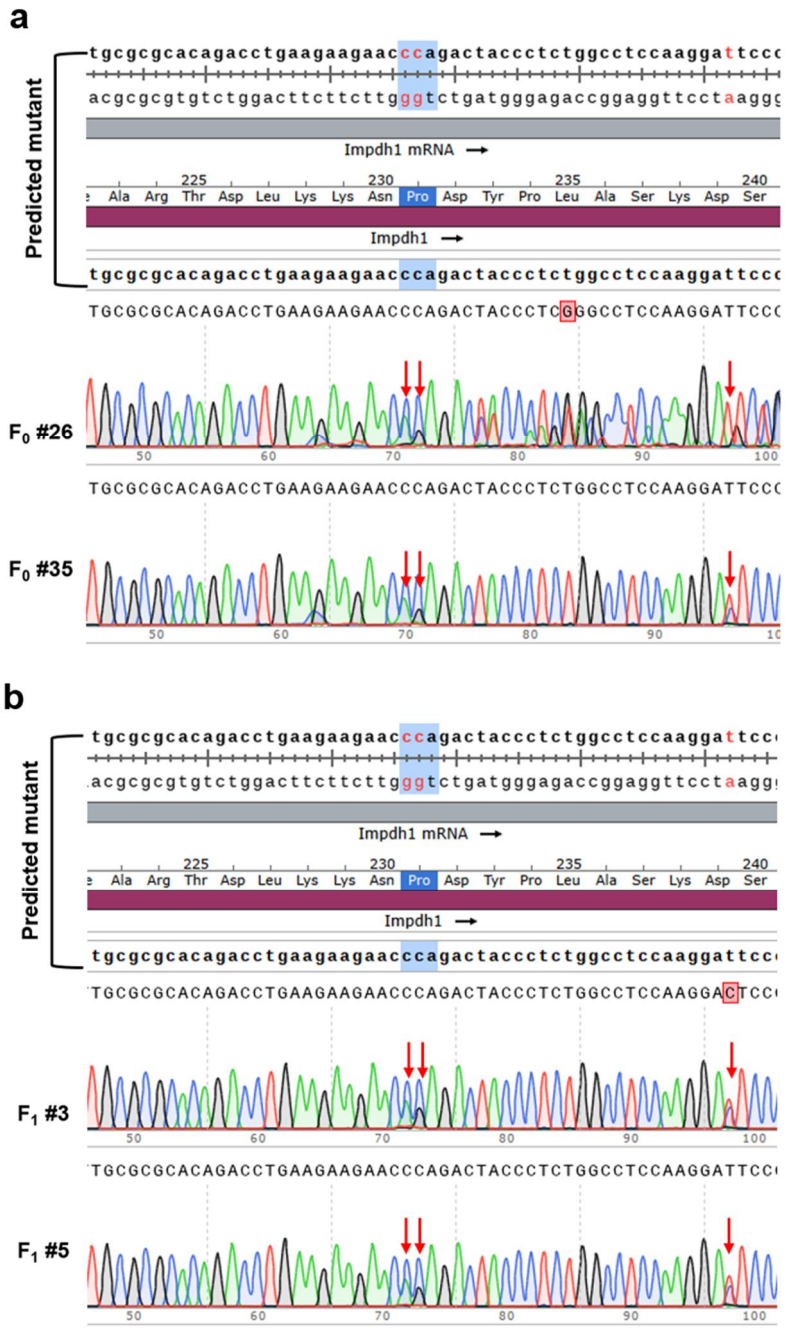
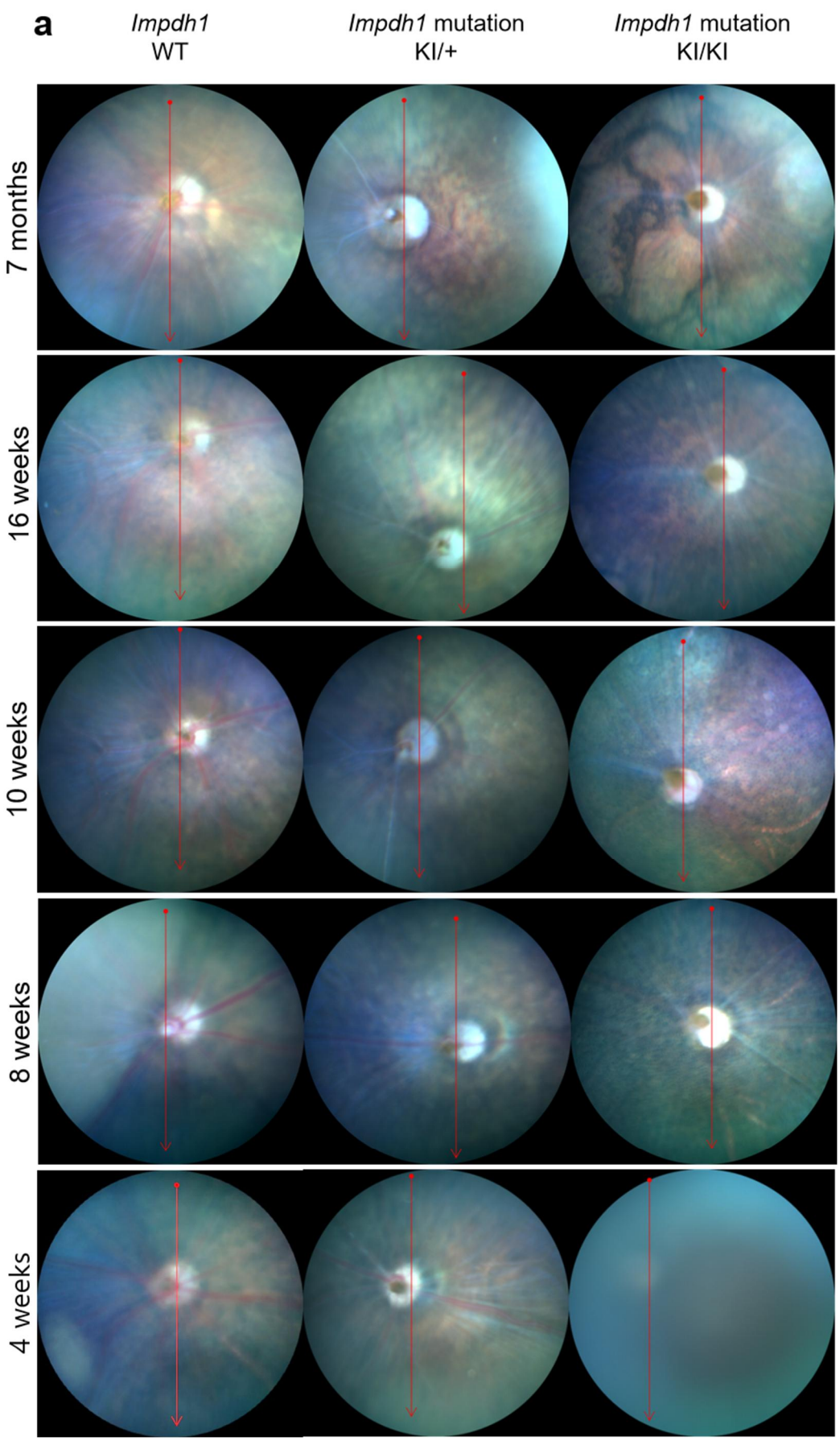
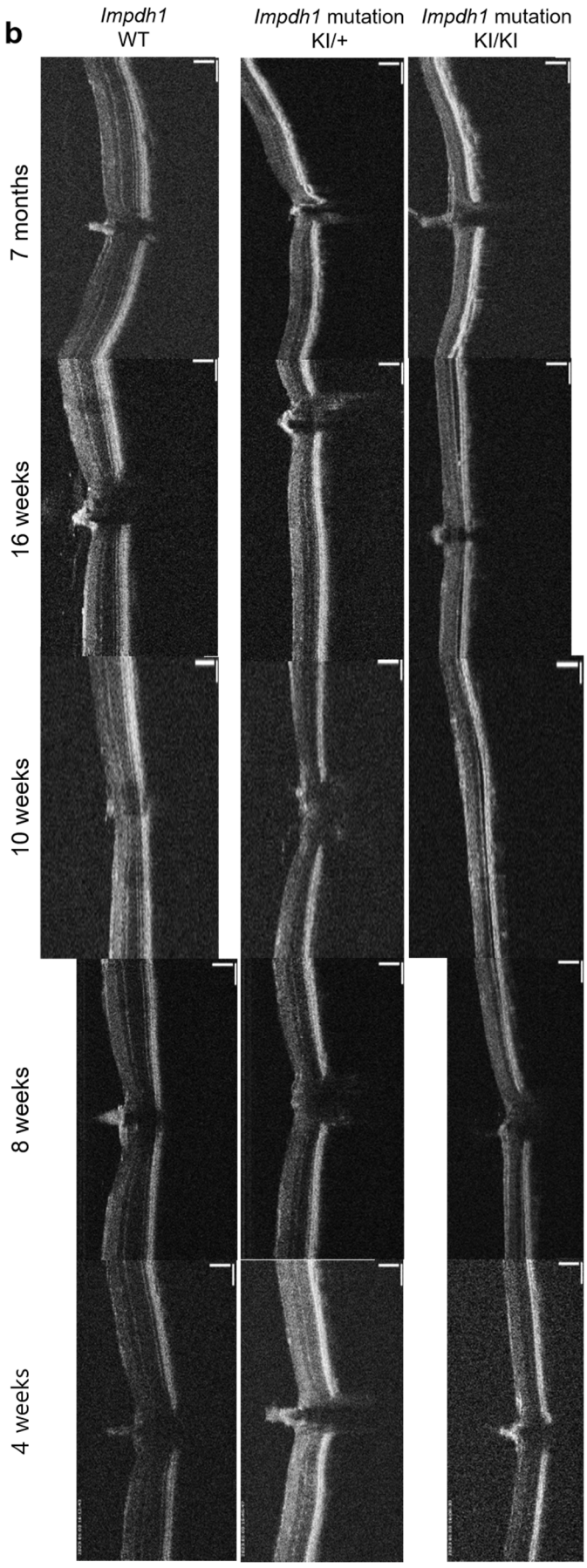


Figure 2. Sequencing results of the IMPDH1-R316P mutant mouse manufactured using CRISPR-Cas9. (a) Sequencing results of the IMPDH1-R316P mutant founder (F0) mouse. **(b)** Sequencing results of the IMPDH1-R316P mutant founder mouse (F1) with the wild mouse.


a
Impdh1
WT
Impdh1 mutation
KI/+
Impdh1 mutation
KI/KI
7 months
16 weeks
10 weeks
8 weeks
4 weeks


b
Impdh1 WT
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Impdh1 mutation KI/KI
7 months
16 weeks
10 weeks
8 weeks
4 weeks

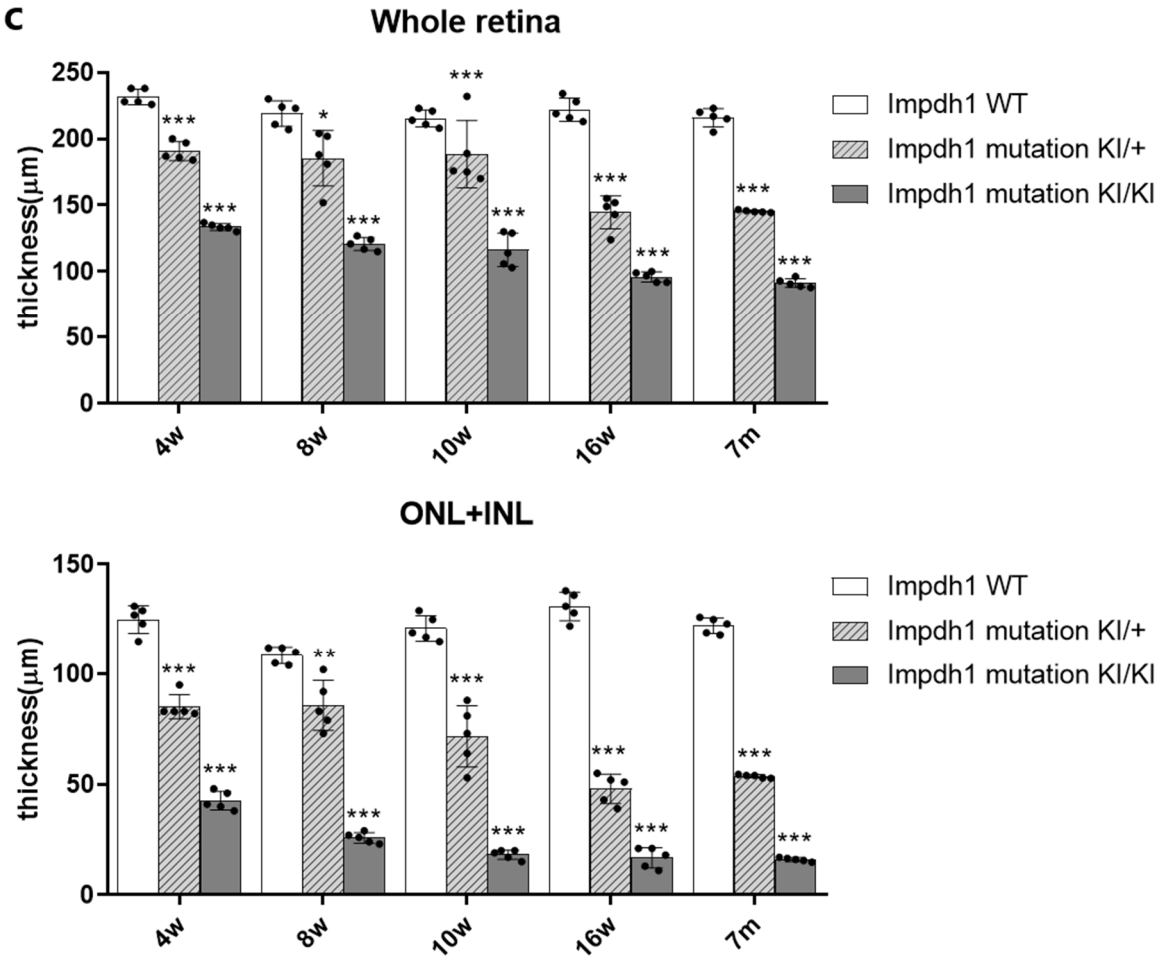

Figure 3. ***In vivo*** **retinal morphologic changes associated with the IMPDH1 mutation. (a)** Optic disc-centered fundus photography. **(b)** Serial infrared optical coherence tomography (OCT) to compare the thickness. **(c)** Retinal thickness change (Whole retina/INL+ONL). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, n = 5 retinas per group.

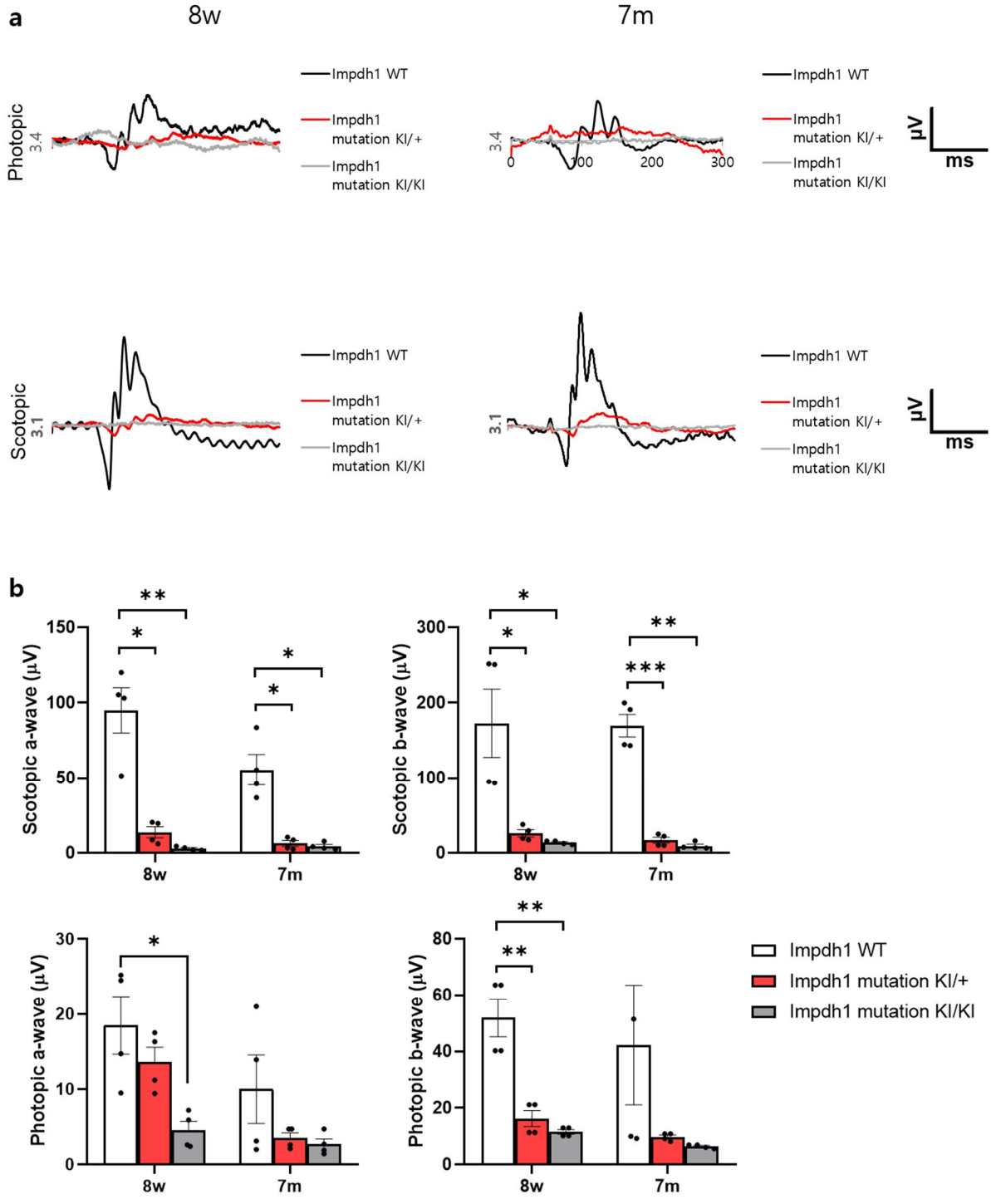

Figure 4. Electroretinogram (ERG) of mice with the IMPDH1 mutation. Representative ERG data showing the amplitudes of both photopic and scotopic responses. (a) Serial infrared optical coherence tomography (OCT) to compare the thickness. **(b)** Quantification of the amplitude of photopic and scotopic a- waves & b-waves. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, n = 4 retinas per group.

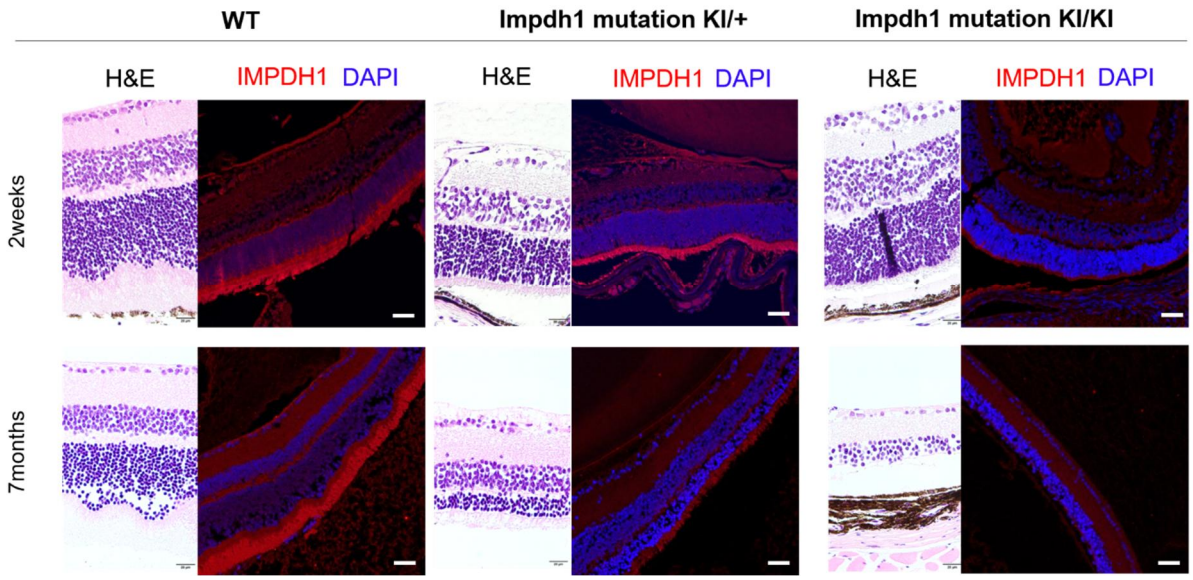

Figure 5. Histologic sections of wild-type, IMPDH1 mutation heterozygote, and IMPDH1 mutation homozygote mouse at 2 weeks and 7 months postnatal age (H&E and immunofluorescence assay). *Scale bars*: 20 μm (H&E), 50 μm (immunofluorescence assay).

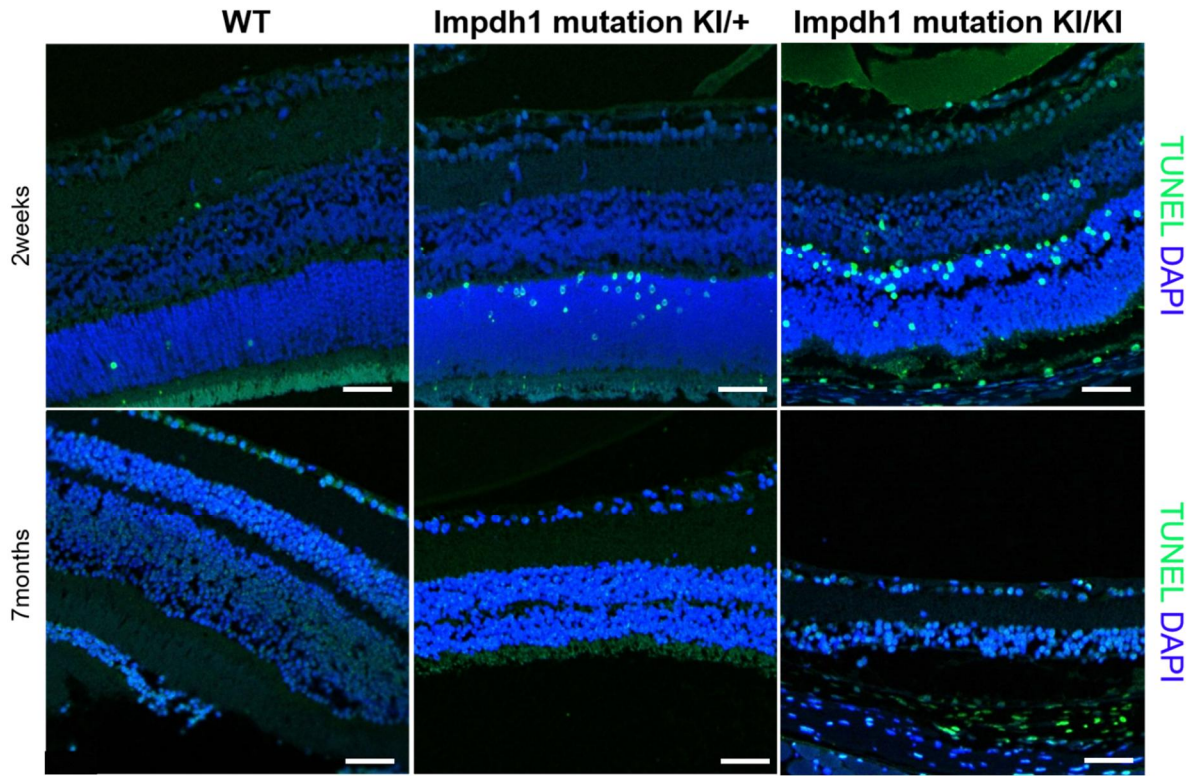

Figure 6. TUNEL staining of wild-type rats and IMPDH1 mutation mouse at each genotype. TUNEL-positive cells fluoresced yellow-green. *Scale bars*: 50 μm.

Discussion

Under the CRISPR-Cas9 system [23], using ssODN, we developed a retinal degeneration model with a human IMPDH1-R316P mutation that exhibits retinal degeneration. We investigated the ophthalmic appearance, time-dependent changes in retinal morphology, and visual functions using fundus photography, OCT, ERG, and histologic analyses. A major finding of this study is that IMPDH1 mutation mice develop retinal degeneration similar to human RP.

In humanized IMPDH1-R316P mice, initial signs of retinal degeneration, which include decreased retinal thickness, were observed at 4 weeks of postnatal age. At 8 weeks of postnatal age, retinal detachment began in homozygotes. In the case of heterozygotes, retinal detachment did not occur. However, the thickness of the retina was significantly reduced compared to the wild type. Retinal thickness begins to thin from ONL, and INL (secondary to photoreceptor degeneration) almost disappears at 7 months.

We performed a TUNEL assay to confirm how photoreceptor apoptosis was expressed according to the genotype of the IMPDH1-R316P mutation: wild-type, heterozygote, and homozygote. At 2 weeks of postnatal age, it was observed that homozygotes and heterozygotes had a lot of cell apoptosis. Cell apoptosis was limited to ONL and occurred in homozygotes much more than heterozygotes. At 7 months of postnatal age, cell apoptosis no longer occurred because most cells of ONL were already dead.

Similar morphologic alterations have been reported in mouse models lacking functional cones and rods [10,11] and in human patients [26]. Among the previously reported RP animal models, mice models include R/rd, rd1, rd4, rd8, rd9, rd10, etc. Depending on the inheritance pattern, P/rd and rd4 are AD, rd9 is XR, and rd1, 8, 10, and 12 are AR. Among them, in the P/rd model, which is AD like our IMPDH1 model, after 3 weeks postnatal age, the photoreceptor cells lacking the outer segment (OS) begin to undergo slowly progressive degeneration, and after 12 months, few cells remain. At P21, rod-mediated ERG presents severely inadequate responses. Furthermore, in rd 4, the ONL decreases at 10 days old and presents total loss at 6 weeks. ERG is poorly detected between 3 and 6 weeks [24]. P/rd progresses slower than our model, and Rd4 progresses faster than IMPDH1 homozygote even when heterozygote.

In human patients, perifoveal photoreceptor loss was measured over the years as constricted and progressing over time. Furthermore, full-field ERGs were severely reduced, and the dark-adapted rod and mixed responses were extinguished at earlier visits than the light-adapted cone responses [3].

These findings suggest that the IMPDH1-R316P mutation KI mice may provide a good model to study the pathogenesis of RP, which is characterized by preferential loss of the photoreceptor layer at an early stage. Moreover, our novel humanized IMPDH1-R316P mutation KI mice robustly recapitulate the hallmark phenotypes of patients with RP and may provide a reliable model for translational research concerning human retinal degeneration.

Various cases have been reported on techniques for manufacturing retinal degeneration animal models using genetic scissors [24,25], and a retinal degeneration rat animal model manufactured by inducing a defect in the pde6b gene using CRISPR-Cpf1 gene scissors is disclosed in previous studies [4]. However, the disclosed retinal degeneration rat animal model knocked out the pde6b gene function through CRISPR-Cpf1 gene scissors technology, and no retinal degeneration animal model has been launched by inducing the replacement of a specific base using ssODN under the CRISPR-Cas9 system. Therefore, we manufactured an animal model with a human IMPDH1-R316P mutation through HDR using ssODN as a donor template and confirmed that the animal model showed significant retinal degeneration in heterojunction mutations and isomorphic junction mutations, which can be used as an animal model for eye diseases caused by retinal degeneration. In addition, the final goal is to provide a screening method for drugs for preventing or treating retinal degeneration using the genetically modified animal model created in this way.

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국문 요약

연구목적

망막색소변성증은 망막 시세포가 손상되어 실명에 이르는 질환으로, 아직 효과적인 치료법이 없다. 따라서 치료방법의 개발을 위해 다양한 치료법을 적용할 수 있는 망막 변성 질환을 가진 실험동물의 개발이 필수적이다. 망막색소변성증을 유발하는 유전자 중에서도 IMPDH1 유전자는 선행연구에서 확인된 망막색소변성 유전 이상 원인 중 가족검사에서 높은 확률로 원인 유전 이상일 것으로 추정되나, 그 병리적 특성이 확실하지 않아 동물 모델을 통한 병리적 임상양상을 확인하고자 IMPDH1 변이 동물모델을 제작하였다.

연구방법

CRISPR-Cas9 시스템 하에 정상 C57BL/6J 마우스에 단일가닥 DNA 올리고 뉴클레오타이드(single-standed oligo DNA nucleotide, ssODN)를 사용하는 단일 염기 치환을 유도하여 인간 IMPDH1-R316P 돌연변이를 갖는 망막 변성 모델을 제작하였다. 제작한 IMPDH1 변이 인간화 마우스는 야생형, 이형접합, 동형접합 세 가지로 나누어 7개월령까지 안저촬영 (Fundus photography)과 빛간섭 단층촬영 (OCT; Optical Coherence Tomography), 조직학적 검사를 통해 각 유전형의 병리적 특성을 확인하였다.

연구결과

2주령까지는 유전자형끼리 큰 차이가 없다가 4주령부터는 망막의 두께 및 층의 선명함에 명확한 차이가 보인다. 야생형의 경우 7개월령까지 정상적으로 층이 잘 유지되며 안저촬영에서도 혈관이 선명한 것을 확인할 수 있으나, 동형접합과 이형접합은 4주령부터 확연히 망막의 두께가 얇아지는 것이 관찰되며 안저촬영에서도 시신경과 혈관 부분이 상당히 창백해진 것을 확인할 수 있다. 8주령쯤 되면 동형접합에서는 망막박리 또한 관찰되었다. 7개월령에서는 동형접합에서는 망막의 층이 거의 남아있지 않고 망막색소변성이 매우 많이 진행되어 얼룩덜룩해진 것이 확인되었다. 이형접합은 동형접합 만큼 심하지는 않으나, 야생형에 비하여 망막이 확연히 얇아져 있고 망막색소변성 또한 많이 진행되어 있는 것을 확인할 수 있다.

결론

제작한 IMPDH1 변이 인간화 마우스의 유전형 (야생형, 이형접합, 동형접합)에 따라 표현형이 다르게 나타나는 것을 확인하였다. 이에 IMPDH1 유전자는 우성 유전 되는 것이며, 유전형에 따라 그 증상의 정도가 달라짐을 확인할 수 있었다.

주요어

망막색소변성증, 우성 유전, 동물모델, 망막시세포, IMPDH1 돌연변이, 인간화 마우스