Missense variants in the conserved transmembrane M2 protein domain of \textit{KCNJ13} associated with retinovascular changes in humans and zebrafish

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A B S T R A C T

Mutations in \textit{KCNJ13} are associated with two retinal disorders; Leber congenital amaurosis (LCA) and snowflake vitreoretinal degeneration (SVD). We describe a novel fibrovascular proliferation in the retina of two affected members of a \textit{KCNJ13}-related LCA family with a homozygous c.458C > T, p.(Thr153Ile) missense mutation. Optical coherence tomography retinal imaging of the \textit{kcnj13} mutant zebrafish (\textit{obelix\textsuperscript{td15} c.502T > C, p.(Phe168Leu)}) revealed a late onset retinal degeneration at 12 months, with retinal thinning and associated retinovascular changes, including increased vessel calibre and vitreous deposits. Both human and zebrafish variants are missense and located within the conserved transmembrane M2 protein domain, suggesting that disruption of this region may contribute to retinovascular changes as an additional feature to the previously described LCA phenotype. Close monitoring of other patients with similar mutations may be required to minimise the ensuing retinal damage.

1. Introduction

The \textit{KCNJ13} gene (potassium voltage-gated channel subfamily J member 13) encodes Kir7.1, a member of the inwardly-rectifying potassium channel family (Krapivinsky et al., 1998; Partiseti et al., 1998; Doring et al., 1998). The Kir7.1 protein consists of a pore domain flanked by two transmembrane regions and functions as a homotetramer. The channel is expressed at the plasma membrane of a variety of ion-transporting epithelia, including the retinal pigment epithelium (RPE) (Kusaka et al., 2001; Hejtmancik et al., 2008; Yang et al., 2008). In the RPE, Kir7.1 is localized to the apical membrane at the interface with the photoreceptor outer segments, where it facilitates potassium ion (K\textsuperscript{+}) efflux to the subretinal space (Yang et al., 2003) and is involved in controlling fluid flow across the blood–retina barrier (Hughes and Takahira, 1996; Doring et al., 1998). Kir7.1 shows co-localization with the Na\textsuperscript{+}/K\textsuperscript{+} pump, suggesting that it is involved in K\textsuperscript{+} recycling required to keep up with high rates of epithelial ion transport (Nakamura et al., 1999).

Mutations in \textit{KCNJ13} have been linked with two ocular disorders: (i) autosomal recessive Leber congenital amaurosis (LCA, MIM #614186) (Sergouniotis et al., 2011; Pattnaik et al., 2015; Perez-Roustit et al., 2017), and (ii) autosomal dominant snowflake vitreoretinal degeneration (SVD, MIM #193230) (Hejtmancik et al., 2008). LCA is a severe early onset retinal dystrophy with RPE and photoreceptor loss causing blindness from birth (Kumaran et al., 2017). It is characterized by sensory nystagmus, amaurotic pupils and absent electrical signals on an electroretinogram. Retinal features including macular atrophy, pigment deposits and vessel attenuation have been reported in LCA patients with \textit{KCNJ13} mutations (Sergouniotis et al., 2011; Pattnaik et al., 2015; Perez-Roustit et al., 2017). SVD is a disorder characterized by a fibrillar vitreous degeneration, peripheral retinal abnormalities including small inner retinal crystalline-like deposits resembling snowflakes and chorioretinal atrophy, optic nerve head dysmorphism, early-onset cataracts and corneal guttae (Hejtmancik et al., 2008). The visual acuity is normal and retinal function testing reveals only mild abnormalities.

Kir7.1 is well conserved and defects in this channel have been found to cause retinal disease in both mice (Zhong et al., 2015; Roman et al., 2016) and zebrafish (Toms et al., 2019). The \textit{obelix} (\textit{obelix\textsuperscript{td15}}) zebrafish mutant, generated through ENU mutagenesis, harbors a missense mutation (c.502T > C, p.Phe168Leu) in \textit{kcnj13}, which affects the transmembrane region abolishing K\textsuperscript{+} conductance by disrupting K\textsuperscript{+} permeation through the channel (Iwashita et al., 2006). Previously, we have described the retinal disease in this zebrafish model which corresponds to the changes seen in patients with \textit{KCNJ13}-related LCA (Toms et al., 2019). Here, we describe retinovascular abnormalities not reported previously in a human family with c.458C > T, p.(Thr153Ile) \textit{KCNJ13} mutations with corresponding retinovascular defects in the...
Table 1

| Patient | Age | Mutation | RVA | LVA | FFA |
|---------|-----|----------|-----|-----|-----|
| A-1     | 20  | c.458C > T, p.Thr153Ile | CF  | NPL |     |
| A-2     | 18  | c.458C > T, p.Thr153Ile | CF  | 1.2 logMAR |     |
| B-1     | 13  | c.458C > T, p.Thr153Ile | 1.3 logMAR | 1.3 logMAR |     |
| B-2     | 10  | c.458C > T, p.Thr153Ile | CF  | CF  |     |

*Hejtmancik et al. (2008) Perez-Roustit et al. (2017) Pattnaik et al. (2015) Khan et al. (2015) Hejtmancik et al. (2008) Lee et al. (2003) RVA, right visual acuity; LVA, left visual acuity; FFA, fundus fluorescein angiogram; CF, count fingers; NPL, no light perception; PRP, pan-retinal photoacoagulation; logMAR, logarithm of the minimal angle of resolution. All patients had LCA except for where marked *SVD family.

obed15 zebrafish mutant suggesting an additional clinical feature of this condition.

2. Materials and methods

2.1. Patient evaluation

The study was approved by the local research ethics committee, and all investigations were conducted in accordance with the principles of the Declaration of Helsinki; informed consent was obtained from all participating individuals. Molecular genetic testing on genomic DNA extracted from blood using an LCA next-generation sequencing (NGS) panel including KCNJ13 (MIM #603208), with Sanger sequencing to confirm the mutations. Ophthalmic evaluation included slit lamp examination, fundus examination and fundus fluorescein angiogram (FFA) with Optos imaging, as part of routine clinical care.

2.2. Zebrafish husbandry

Wild-type AB and obed15 zebrafish were generated by natural pairwise matings of genotyped homozygous or heterozygous fish and raised at 28.5 °C on a 14 h light/10 h dark cycle in the UCL zebrafish facility. Zebrafish were maintained according to local UCL and UK Home Office regulations for the care and use of laboratory animals under the Animals Scientific Procedures Act at the UCL Institute of Ophthalmology animal facility. UCL Animal Welfare and Ethical Review Body approved all procedures for experimental protocols, in addition to the UK Home Office (License no. PPL, PC916FDE7). All zebrafish experimentation followed the ARRIVE guidelines for animal research.

2.3. Optical coherence tomography (OCT) zebrafish imaging

Wild-type and obed15 retinas were scanned using the Bioptigen Envisu R2200 Spectral Domain Ophthalmic Imaging System (Bioptigen, Inc.) as described (Toms et al., 2017). The approximate same region of dorsal retina was imaged in each zebrafish using a 1 × 1 mm perimeter scan with 400 A-scans per B-scan with 400 total B-scans. To assess anatomical vessel differences, en face slabs were generated using custom matlab software (Mathworks, Inc). These slabs were defined at 10 μm below the inner limiting membrane to 40 μm above. This provided a map of the retinal vasculature. Measurements of vessel width were extracted using longitudinal reflectivity profiles (LRP) (Huang et al., 1998). Distance from the edge of the optic nerve was also calculated. If the optic nerve was not visible in the scan, distance from the optic nerve was calculated using the angles of the vessels. Measurements were taken from 3 to 7 locations per fish. The slopes of vessel width by location were compared between the wild-type and mutant zebrafish. A multi-linear model for mixed effect testing was used for statistical analysis, which was performed using JMP13 (SAS Institute Inc).

3. Results

3.1. Retinal vasculature changes in patients with KCNJ13-LCA

Two unreported and unrelated LCA families, diagnosed in infancy, were found to carry the same homozygous missense mutation c.458C > T, p.(Thr153Ile) in KCNJ13 (Table 1, Fig. 1). This variant was previously identified in the supplementary data from Lee et al. (2014). This change is located in exon 2 of the gene within the coding region for the M2 transmembrane domain of Kir7.1. The c.458C > T, p.(Thr153Ile) variant was found to have a CADD score of 26.8 (damaging) and PolyPhen2 score of 1 (probably damaging). It is also predicted to be damaging/disease-causing by SIFT and MutationTaster.

Both families were investigated for retinochoroidal changes. The affected siblings in family A developed retinal neovascularization with diffuse severe leakage and vitreous haemorrhages at age 17 on a background of characteristic KCNJ13-LCA (with areas of nummular pigment at the level of the RPE, especially over the posterior pole, macular atrophy and optic disc pallor with retinal vessel attenuation). The initial appearances of the retinas in the two brothers looked fairly typical of LCA with extensive RPE changes in both the macula and retinal peripheries with progressive pigment migration and vessel attenuation (Fig. 2a–d). However, prior to the development of the vitreous haemorrhage, there was preretinal fibrosis over the disc and along the arcade. Unlike typical foci of neovascularization, there was diffuse leakage from the retinal vessels and a diffuse vaso-proliferative response. OCT imaging showed that the retinal layers lacked normal segmentation and there was no evidence of a foveal depression or pit (Fig. 2e).

Patient A-1 developed fibrosis along the arcades and optic disc; they...
received extensive pan-retinal photocoagulation (PRP) and two intravitreal injections of avastin. Following a left vitreous haemorrhage, the patient had a pars plana vitrectomy with secondary complications of cataracts requiring phacoemulsification followed by uveitic glaucoma resulting in posterior capsule opacification and membranectomy. Patient A-2 also developed retinal neovascularization and right vitreous haemorrhage requiring extensive PRP. FFA was performed to investigate for ongoing retinal neovascularization and early and late leakage is seen in both patient retinas (Fig. 3d–f). FFA findings from the normal control subject were typical of a healthy retina (Fig. 3a–c) (Singer et al., 2016).

In comparison, affected siblings in family B showed characteristic features of LCA with patient B-1 showing very mild leakage from the optic disc in both eyes, but no evidence of retinal neovascularization elsewhere on FFA. Patient B-2 showed no retinovascular changes (Fig. 3g–i). However, both patients are younger than those from family A and annual monitoring is required. In the previous families diagnosed with KCNJ13-LCA, where affected patients are between 10 and 34 years, no previous reports of retinovascular changes have been described (Table 1).

3.2. Retinal vasculature changes in the obetd15 zebrafish

The previously described obetd15 zebrafish showed a late onset retinal degeneration with retinal thinning and disruption of the photoreceptor and RPE layers, which became apparent at 12 mpf (Toms et al., 2019). The obetd15 mutation is c.502T > C, p.Phe168Leu, located within the M2 domain coding region (Fig. 1c); when the human and zebrafish Kir7.1 protein sequences are aligned, the amino acid changes carried by the obetd15 fish and LCA patients are separated by one residue. OCT imaging at 12 mpf revealed gross abnormalities in the appearance of the inner retinal vasculature of obetd15 zebrafish (Fig. 4). Comparison of age-matched wild-type and obetd15 central retinal vessels emerging from the optic nerve region show that the vessels appeared to be abnormally enlarged and hyper-reflective in the obetd15 retina compared to wild-type, allowing them to be much more easily distinguished on OCT en face projections (Fig. 4a and b). The difference in vessel thickness was also apparent on the B-scan cross-sectional images where they appear as prominent spherical structures overlaying the ganglion cell layer in the obetd15 retina while relatively unremarkable in comparison on the wild-type images (Fig. 4c and d). Analysis of vessel width as a function of distance from the optic nerve showed a significant
correlation ($p < 0.001$) between width and distance from the optic nerve head (Fig. 3g). Measurements were taken from 3 wild-type and 5 obetd15 zebrafish. The retinal vessels of obetd15 zebrafish were consistently wider and slopes of the trend lines were significantly different ($p < 0.0001$), suggesting that the mutant vessels were wider for a given distance from the optic nerve compared to wild-type controls. OCT imaging of heterozygous obetd15 zebrafish showed retinal vasculature comparable to that of wild-type fish (Supplementary Fig. S1).
In addition, examination of two regions at level of the vessels and just above the vessels on volume intensity projection images revealed the presence of fibrous material in the obetd15 vitreous which was not present in the same regions on wild-type images (Fig. 4c and d). Spot-like hyper-reflective vitreous deposits were also noted on both B-scan and en face views of the obetd15 retina (Fig. 4e and f).

4. Discussion

We have described here retinovascular abnormalities in two siblings (A-1 and A-2) with homozygous c.458C > T, p.(Thr153Ile) KCNJ13 mutations. Both patients showed features characteristic of LCA, including chorioretinal atrophy and nummular pigmentary retinopathy. In addition, early and late leakage of retinal vessels with neovascularization and vitreous haemorrhage was observed, which have not been noted in KCNJ13-LCA previously (Sergouniotis et al., 2011; Pattnaik et al., 2015; Perez-Roustit et al., 2017). These abnormalities were unlike vasoproliferative tumors, Coats-like retinopathy or the SVD phenotype. Because there is consanguinity within both of these reported families, one might suspect that perhaps another homozygous genetic variant shared between the two brothers might be responsible for their retinovascular complications. However, our findings in the zebrafish model strongly suggests that the KCNJ13 variants alone could be responsible for this clinical feature. These novel findings within our patients and the animal model strongly suggest that this retinovascular complication should be considered within the clinical spectrum of KCNJ13-related retinal dystrophy (rather than as an incidental and independent finding) and perhaps dependent on the specific nature and position of the mutation and its impact on protein function.

The retinal degeneration seen in the KCNJ13 model zebrafish,

![Fig. 4. Retinal vasculature in obetd15 zebrafish.](image-url)

OCT en face images of the dorsal retina show inner retinal vessels emerging from the optic nerve region in the wild-type (a) and obetd15 (b) fish at 12 months post-fertilization, displaying abnormal blood vessel appearance in the mutant retina. Cross-sectional B-scans from wild-type (c) and obetd15 (d) fish also demonstrate notable differences in vessel size (vessels indicated with *). Numbered brackets (1–4) on the cross-sections represent areas analyzed in the corresponding en face images. Fibrous material was apparent at each depth examined in the vitreous of the obetd15 retina (3, 4) but was not seen on the wild-type images (1, 2). Hyper-reflective deposits were also noted in the vitreous of the obetd15 retina on both B-scan (e) and en face (f) images, indicated with arrows. Graph (g) shows retinal vessel thickness plotted against distance from the optic nerve in wild-type and obetd15 zebrafish. Scale bars = 50 μm.
Several retinal vascular and pigment changes and deposits in the vitreous are characteristic in patients with SVD (Lee et al., 2003), and hyper-reduction of the vitreous is a common finding in retinovascular abnormalities in the obes1 mice (Toms et al., 2019), showing significant retinal vascular abnormalities. This could mean that the retinovascular abnormalities might manifest itself from teenage years onwards or there could be other genes that play a role in the expression of this phenotype. For affected members of the family who have the same mutation but no vascular changes, regular monitoring would be recommended as they are not yet of the same age and the onset may be forthcoming.

In summary, we have reported novel retinovascular findings in both human LCA patients and a corresponding zebrafish mutant, associated with KCNJ13 missense mutations. Further experimentation will be necessary to determine any involvement of Kir7.1 in vascular growth and function.

Declaration of competing interest
None.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.exer.2019.107852.

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