

s/m² with a single flash. To isolate cone response, eyes were light adapted for 10 minutes with a 25cd/m² adapting field. Cone ERGs were obtained using flash intensity 2.50cd·s/m² presented at 0.5Hz with 50 flashes averaged for this intensity. Electrical signals were amplified with 8,300 gain and a bandwidth of 0.5 to 500Hz, electrical signals were digitized at a 10kHz rate using Evoked Potential/EMG Measuring System (Neuropack μ MEB-9102, Nihon Kohden Corporation, Tokyo, Japan).

Immunohistochemistry

The eyes were sectioned at 5 μ m thickness along the vertical meridian through the optic nerve head. After deparaffinization and rehydration, the tissue sections were treated with Target Retrieval Solution (DakoCytomation, Denmark). The sections were incubated with blocking solution for 1 h followed by overnight incubation with primary antibody against HA tag (1:500 dilution; Sigma-Aldrich, St. Louis, MO), tublin β III isoform (1:100 dilution; Millipore, Billerica, MA), OPTN (1:500 dilution; kind gift from Mansoor Sarfarazi), or Calretinin (1:500 dilution; Sigma) in PBS containing 1% BSA at 4°C. Slides were washed in PBS and then incubated with Alexa 488 or Alexa 568 (1:500 dilution; Invitrogen) conjugated secondary antibody and with 4', 6'-diamidino-2-phenylindole (DAPI) for nuclear staining for 1 h at room temperature.

The stained tissues were examined using confocal fluorescence laser microscope (Radiance 2000, Bio-Rad Laboratories, Hercules, CA). For negative control of the immunohistochemical staining, the sections were incubated with blocking solution without primary antibody (data not shown).

Whole-mount immunostaining

The whole-mount immunostaining was performed essentially as described (Jakobs et al., 2005). Anterior parts were dissected from enucleated eyes. The posterior parts were fixed in 4% PFA/PBS for 2 hours on ice and then incubated with the anti-SMI32 (1:200 dilution; Sternberger Monoclonals, Baltimore, MD), anti-GFAP (1:200 dilution; Millipore) or anti-active caspase-3 (1:200 dilution; Imgenex, San Diego, CA) antibody for 7 days at 4°C. Slides were washed in PBS containing 0.1% Triton X-100 and then incubated with Alexa 488 or Alexa 568 (1:500 dilution; Invitrogen) conjugated secondary antibody and with 4', 6' -diamidino-2-phenylindole (DAPI) for nuclear staining for 2 days at 4°C. The retinas were then mounted with Vectashield (Vector Laboratories, Burlingame, CA) and evaluated on a confocal microscope.

Measurement of intraocular pressure (IOP)

The average IOP for each genotype was recorded. IOP was measured using an

impact-rebound tonometer (Colson Medical Supply, Franconia, NH) and optical interferometry tonometer (FISO Technologies, Quebec, Canada) for mice of each genotype as described (Senatorov et al., 2006). Using the rebound tonometer, we were able to measure IOP in awake and non-sedated mice of various ages, while optical interferometry tonometry was performed on anesthetized animals. Measurement of IOP was always performed in the morning between 10 and 12 a. m. The numbers of mice successfully assessed for each genotype and age were 18 weeks and 12 month.

Measurement of OPTN-Rab8 interaction

OPTN-Rab8 interaction analysis was performed using *CoralHue*[®] Fluo-chase Kit (MBL, Tokyo, Japan). Based on the instruction manual, we constructed *OPTN* wild type, *OPTN* E50K, and Rab8 wild type, Rab8 GDP-bound (T22N) inactive form, and Rab8 GTP-bound (Q67L) active form recombinant fusion protein plasmids with both N-terminal and C-terminal fluorescence protein (Fig. 7A). RGC-5 and COS1 cells were transfected by each pairs of the plasmid mixtures using Fugene HD (Roche). 48 h after transfection, the medium was replaced to phosphate buffered saline (PBS) and the cells were observed with inverted microscope (Eclipse TE300, Nikon). To observe OPTN-Rab8 complex, cells were fixed 48 h after transfection with 4%

paraformaldehyde in PBS on ice for 20 minutes. Cells were incubated in blocking buffer (3% bovine serum albumin, 0.1% Triton X-100 in PBS) and then with anti-GM130 antibody (BD bioscience, San Jose, CA) at room temperature for 1 h each. Cells were washed three times with PBS-T (0.1% Triton X-100 in PBS) and then incubated with Alexa-568 conjugated secondary antibody for 1 h at room temperature. Slides were washed, mounted, and analyzed by confocal microscopy.

Measurement of OPTN-Rab8 interaction by quartz crystal microbalance (QCM)

The OPTN-Rab8 interaction was examined by highly sensitive 27MHz quartz crystal microbalance (QCM) (Hama et al., 2004). Rab8, OPTN, and OPTN E50K were cloned into expression vector pTrcHisB and transformed into E. coli strain BL21. Protein extracts were applied to equilibrated 1 ml Ni-chelate affinity column (Qiagen, Hilden, Germany) for purification. The gold plate on the QCM sensor chip (Initium, Tokyo, Japan) was cleaned with a mixture of sulfuric acid and hydrogen peroxide (1:3) for 5 min followed by immediate washing with water. One hundred microliters of 0.1 M glycine-HCl (pH 2.4) was then applied to the gold plate and incubated for 10 s. The sensor chip was washed again and covered with water until 150 μ g of Rab8 was bound to the gold plate. Rab8 bound sensor chip was dipped into 8 ml reaction chamber containing 25% blocking buffer in PBS. Sensor chip vibration was monitored

until stabilized, then less than 8 μ l of purified wild type or mutant OPTN were injected into the reaction chamber. A new sensor chip bound with Rab8 was used for each OPTN variant.

GST pull-down assay

Rab8 cDNA was cloned into expression vector pET42b and expressed in *E. coli* strain BL21 (DE3) to produce glutathione-S-transferase (GST) fused Rab8 protein. Supernatant fraction containing His-OPTN and His-OPTN E50K were incubated with GST and glutathione-Sepharose beads at 4°C for 30 min with continuous agitation. Each supernatant was then incubated with GST alone or GST-Rab8 with glutathione-Sepharose beads. The beads were then washed 3 times with binding buffer before eluting the bound proteins with buffer containing 20 mM glutathione. Eluted samples were analyzed by SDS-PAGE followed by western blotting using anti-Xpress tag antibody (Invitrogen, Carlsbad, CA).

The ocular coherence tomography analysis of patients with OPTN E50K mutation

Four NTG patients with severe visual field loss were identified in a three generation family by the Department of Ophthalmology of Gifu University Graduate School of Medicine. Affected 48 years old female (patient 2) was compared with

unrelated unaffected male (45 yrs) for ocular coherence tomography analysis (OCT2000, Carl Zeiss Meditec, Dublin, CA).

Statistical analysis

All data were expressed as the mean \pm standard deviation (SD). Statistical analysis was performed using Scheffe's procedure after the ANOVA test for multiple comparisons of mean values. $P < 0.05$ was considered statistically significant.

RESULTS

Development of NTG transgenic (TG) mice

The chicken beta-actin promoter (pCAGGS) with CMV enhancer was used for transgene expression and was kindly provided by Dr. Junichi Miyazaki of Osaka University. HA-tag was inserted at the N-terminus of OPTN constructs for detection of proteins expressed by the transgene. Five independent Tg mice lines overexpressing *OPTN* wild type (Wt Tg), mutant E50K (E50K Tg), mutant 1st leucine zipper deletion (1st LZ del), mutant 2nd leucine zipper deletion (2nd LZ del), and mutant H486R (H486R Tg) were developed using the BDF1 and C57BL/6J strain (Fig. 1A). In the *OPTN* E50K mice, early symptoms were observed after 16 month leading to abnormal curvature around the optic disc (Fig. 1B). The distribution and protein expression level

of endogenous and mutant OPTN in the retina are shown as immunostaining with OPTN antibody kindly given by Dr. Mansoor Sarfarazi of University of Connecticut and anti-HA tag antibody respectively (Fig. 1C). Both endogenous and mutant OPTN was detected ubiquitously in all retinal layers at approximately 2-fold for all transgene constructs (data not show).

Loss of RGCs in E50K Tg mice

Paraffin eye sections were stained with hematoxylin-eosin to analyze morphological changes in the retina of Tg mice (Figure 2A). *OPTN* wild type (Wt Tg), 1st leucine zipper deletion (1st LZ del), 2nd leucine zipper deletion (2nd LZ del), and mutant H486R (H486R Tg) mice did not show any abnormalities after 16 months. However, expression of E50K mutant (458 G>A) produced significant changes in the eye. In 16 month old mice, retinal thickness and RGC number were reduced by approximately 43% and 20%, respectively in *OPTN* E50K Tg as compared with normal animals (Fig. 2B). Kinetics of RGC loss and retinal thinning for E50K Tg mice are shown in Fig. 2C. Statistically significant RGC loss and retinal thinning were detected 12 months after birth (Fig. 2C). To determine whether retinal function is affected by the thinning of retinal layers, electroretinograms (ERG) were obtained. E50K Tg mice exhibited abolition of the negative wave that normally follows the

transient b-wave, which originates from RGCs and is termed the photopic negative response (PhNR) (Fig. 2D arrow). The a-wave and b-wave were normal in E50K Tg mice. We concluded that E50K was the only mutation among the mutations made expression of which could lead to significant abnormality of retinal histology and function.

Synapse disruption and RGC cell death in OPTN E50K mice

Immunostaining for calretinin, a specific marker for RGCs and amacrine cells, showed loss of the synapses of these cells in the inner plexiform layer (IPL) of 16 month OPTN E50K Tg mice (Fig. 3A arrow). We confirmed excavation of the optic nerve head and immunostaining with anti-tubulin β III antibodies confirmed significant thinning of the nerve fiber layer in E50K Tg mice (Fig.3B arrow). Considering the abundant expression of mutant *OPTN* E50K in all retinal layers, these data indicate that RGC and nerve fiber layer are more sensitive to the expression of E50K *OPTN* and this may be the cause for NTG phenotype.

RGC and glia cell loss in OPTN E50K retina

The whole mount retina was immunostained for SMI32, a neurofilament marker for alpha-type RGCs. The number of RGCs and length of extending axons in *OPTN*

E50K Tg mice were significantly reduced compared to normal or *OPTN*Wt Tg mice (Fig. 4). Double staining for SMI32 and activated caspase-3 revealed apoptotic cells above the RGC layer in *OPTN* E50K Tg mice (Fig. 5A, B). These caspase-3 positive cells were also positively stained for glial fibrillary acidic protein (GFAP) (Fig. 5C-E), a marker for astrocytes. Interestingly, the apoptotic astrocytes were densely observed in the peripheral (Fig. 5F) compared to the central region of retina (Fig. 5G). The location and the density of apoptotic astrocytes matched the apoptotic localization of retinal neurons resulting as thinning of the peripheral retina (Fig. 2A).

IOP measurement of OPTN E50K Tg mice

IOP was measured using two different non-invasive techniques: an impact-rebound tonometer at wake condition and an optical interferometry tonometer utilizing a fiber optic pressure sensor at anesthetized condition (Fig. 6). All measurements were done between 10:00 AM and noon to minimize the influence of diurnal fluctuation of IOP. The average IOP reading from both devices gave similar IOP for mutant and wild-type mice in the normal range of 15 ± 1 mm Hg for all examined ages. These results demonstrated that glaucoma-like pathological features of mutant E50K Tg mice are not due to changes in IOP.

Disruption of OPTN-Rab8 interaction by E50K mutation

Previous studies have demonstrated that OPTN is involved in a number of cellular processes through interaction with multiple proteins. E50K mutation is located near the N-terminus of the protein where the predicted binding site of Rab8 is located (Hattula et al., 2000) suggesting a possible alteration of OPTN-Rab8 interaction in *OPTN* E50K mutants. To test this hypothesis, we compared the protein-protein interaction of OPTN (Wt, E50K) with Rab8 (Wt, T22N inactive GDP-form, and Q67L active GTP-form) in RGC5 and COS1 cells using *CoralHue*[®] Fluo-chase Kit. We observed over 4-fold greater interaction between wild-type OPTN and the active form of Rab8 compared with the inactive form of Rab8. No interactions between OPTN E50K and all forms of Rab8 were observed (Fig. 7B). This observation was further confirmed by QCM interaction assay and co-immunoprecipitation assay (Fig. 7C). Interaction between host (Rab8) and guest (OPTN) protein was measured by the decrease in frequency of quartz crystal by increase of total mass (OPTN + Rab8). Purified Rab8 was fixed on the QCM sensor chip for measurements of interaction with OPTN Wt and mutant OPTN E50K. As shown in Figure 7C, interaction with Rab8 was completely disrupted by E50K mutation. These results were further supported by the glutathione S-transferase (GST)-pull down experiment using Rab8-GST fusion protein and OPTN Wt and OPTN E50K (Fig. 7D). Increased band intensity was observed for

OPTN wild-type compared to OPTN E50K, showing that the wild type form engaged in a stronger interaction with GST-Rab8 protein.

Localization of OPTN-Rab8 complex

CoralHue[®] Fluo-chase Kit was also used for the localization of OPTN-Rab8 in two cell lines, RGC5 and COS1. Co-immunostaining of OPTN-Rab8 complex with Golgi marker GM130 clearly indicated that the interaction take place adjacent to the Golgi network (Fig. 7E).

Optical coherence tomography (OCT) analysis of patients with OPTN E50K mutation

A family with 4 NTG patients carrying the *OPTN* E50K mutation through three generations was identified by the Department of Ophthalmology at Gifu University School of Medicine (Fig. 8A). All patients have severe visual field defects at normal IOP between 9-19 mm Hg for both eyes (Fig. 8). OCT analysis was performed to evaluate retinal abnormality. Thinning of the retina, especially retinal nerve fiber layer (NFL) and ganglion cell layer (GCL) was observed in patients compared to controls (Fig. 8). The phenotype of the *OPTN* E50K Tg mice mimics clinical features of the patient 2.

DISCUSSION

We report the first NTG mouse model that was developed using the *OPTN* gene mutation found in NTG patients. Similar to human NTG patients, the *OPTN* E50K Tg mice show progressive RGC loss in the peripheral retina without elevation of IOP. *OPTN* Wt, 1st LZ deletion, 2nd LZ deletion, and H486R Tg mice did not show any sign of abnormality even after 16 month. Tg mice lines expressing *OPTN* E50K showed significant thinning of the NFL and INL (Fig. 2, 3, 4). Expression of transgene driven by ubiquitous chicken beta-actin promoter with CMV enhancer gave similar distribution of mutant *OPTN* as the endogenous *OPTN* (Fig. 1C). Considering the fact that mutant *OPTN* is expressed over the entire retina, it is noteworthy that the cell death occurred mainly in the RGCs and astrocytes. These cells may be more sensitive to deleterious effects of *OPTN* E50K expression than other retinal cells and this may be the reason for the NTG phenotype. The amount of RGCs was reduced by 20 % in *OPTN* E50K Tg mice as compared with Wt 16 month old animals. Slow progression of RGC death resembles that in human glaucomatous patients.

Retinal function was shown to be disrupted by *OPTN* E50K mutation. The amplitude of the photopic negative response (PhNR) by E50K Tg mice was marked as slow negative wave after the b-wave often being replaced by a prolonged positive phase above base line (Fig. 2D). The PhNR was probably primarily a reflection of the

spiking activity of RGCs although other spiking cells in the inner retina, such as amacrine cells, may also contribute to PhNR. These data agree with previous human ERG studies showing PhNR disturbances in glaucoma patients (Rangaswamy et al. 2007; Machida et al. 2008).

Surprisingly, apoptotic astrocytes were more abundant in the NFL of the peripheral retina and reduced around the optic nerve head (Fig. 5F, G). Previous studies have shown activated astrocytes in the glaucomatous retina and optic nerve lamina region (Wang et al., 2002; Howell et al., 2007). Glial cells are important structural and functional components of the nervous system, including the retinal astrocytes which originate embryologically in optic nerve and subsequently migrate to their final location in the whole retina (Watanabe and Raff, 1988). It is still unclear whether which cell type, an astrocytes or retinal ganglion cells, is affected before the other. Future study using this animal model may provide new information on why peripheral retinas are more affected in human glaucoma.

OPTN was discovered as the first gene shown to be associated with NTG (Rezaie et al., 2002). The molecular mechanism leading to the disease is still unknown. *OPTN* E50K mutation at the N-terminus of the protein is responsible for severe NTG while H486R mutation at the C-terminus is responsible for JOAG with elevation of IOP (Stone et al., 1997; Willoughby et al., 2004). Differences of phenotype observed for

mutations located in different parts of OPTN protein may originate in the modifications of different protein binding sites. OPTN was originally reported as the second protein (FIP-2) interacting with adenovirus protein E3-14.K (Li et al., 1998) and utilizing TNF-alpha or Fas-ligand pathways to mediate apoptosis, inflammation, and vasoconstriction (Sarfarazi et al., 2003). OPTN is known to interact with a number of proteins to perform multiple cellular functions: Rab8 for vesicle trafficking, Huntingtin for membrane trafficking, transcription factor IIIA and FOS for transcriptional activation (Rezaie et al., 2002; Hattula et al., 2000). Because OPTN is capable of interacting with multiple proteins, it is feasible that the E50K mutation can cause glaucoma by functionally affecting one or more protein interactions. Due to the location of the E50K mutation at the N-terminus of the OPTN protein, we speculated that interaction with Rab8 would be affected. Rab8 belongs to a family of small GTP-binding proteins which act as regulators of multiple cellular processes. Rab GTPases regulate all stages of membrane trafficking, including vesicle transport, cargo sorting, transport, tethering, and fusion (Zerial et al., 2001). Rab8 has been shown to be involved in polarized membrane transport and regulation of vesicular transport from the trans-golgi network (Huber et al., 1993). Docking and fusion of rhodopsin is impaired in photoreceptors by mutated Rab8 (Moritz et al., 2001), while treatment with antisense oligo to Rab8 inhibited membrane traffic in hippocampal neurons leading to

inhibition of neurite outgrowth. The Rab8 GTPase regulates apical protein localization in intestinal cells and cooperates with Bardet-Biedl syndrome proteins to promote ciliary membrane biogenesis (Sato et al., 2007; Nachury et al., 2007). Recently, OPTN was demonstrated to protect survival of NIH3T3 cells under oxidative stress by relocating to the nucleus in a Rab8-dependent manner, while OPTN E50K lost the ability to translocate itself to the nucleus (De Marco et al., 2006). OPTN was shown to be a link between Rab8 and myosin VI to Golgi complex. It plays a central role in Golgi ribbon formation and exocytosis (Sahlender et al., 2005). These data suggest that OPTN-Rab8 interaction is essential for Rab8 function and that disruption of Rab8-OPTN interaction may cause irregular transport within the cells. Gene mutations observed in NTG patients including E50K were located near the first zinc-finger domain which was predicted to be a binding site for Rab8 (Hattula et al., 2000). Additionally, glutamic acid at amino acid 50 is well conserved in 8 different species. Our data demonstrate that E50K mutation abolished interaction of OPNT with all forms of Rab8 on the Golgi complex.

Finally, we identified a three generation family with 4 NTG patients, all of whom carry the OPTN E50K mutation (Fig. 8). OCT analysis demonstrated morphological abnormality at the NFL, GCL and IPL in NTG patients as seen in OPTN E50K Tg mice (Fig. 8). These observation follow previous glaucoma reports showing thinning of the

NFL, GCL, IPL, and INL even before the detectable visual field changes occur (Tan et al., 2008; Guedes et al., 2003; Medeiros et al., 2005; Wassle et al., 1989).

In summary, we have proven that the E50K mutation in the *OPTN* gene can lead to NTG in mice. Although further studies are required to fully understand the detailed mechanism of disease progression, we obtained basic information about the affects of E50K mutation at molecular, cellular, and tissue levels. Due to the fact that *OPTN* interacts with multiple proteins involved in multiple levels of regulation, it is likely that different mutations in the *OPTN* gene may affect diverse cellular pathways. If Rab8 function is altered by *OPTN* mutation, it is likely that exocytosis of proteins regulated by the vesicle transport are affected in RGC or astrocytes. Following this path, the next question would be whether such alteration of protein secretion really exists, and what protein(s) would be involved. If these proteins can be identified, it may serve as potential therapeutic approach to treat patients with *OPTN* E50K mutation.

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