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CLINICAL INVESTIGATION

Survey of microphthalmia in Japan

Sachiko Nishina · Daijiro Kurosaka · Yasuhiro Nishida · Hiroyuki Kondo · Yuri Kobayashi · Noriyuki Azuma

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Abstract
Purpose To report the current status of patients with microphthalmia based on a cross-sectional survey of patient hospital visits.
Methods A questionnaire was sent to the departments of ophthalmology in 1,151 major Japanese hospitals to survey the following: the number of patients with microphthalmia who visited the outpatient clinics between January 2008 and December 2009; gender; age; family history; associated ocular anomalies; complications and systemic diseases; surgical treatment; vision and management. A retrospective quantitative registry of 1,254 microphthalmic eyes (851 patients) from 454 hospitals (39.4%) was compiled.
Results Of the patients for whom data were available, 50% ranged in age from 0 to 9 years. The major ocular findings were nanophthalmos, coloboma, and vitreoretinal malformations. Ocular complications frequently developed, including cataracts, glaucoma, and retinal detachment. Surgery was performed in 21.4% of all cases, and systemic diseases were present in 31% of all cases. The vision associated with microphthalmia exceeded 0.1 in about 30% of the eyes. Glasses and low vision aids were used by 21.6% of patients.
Conclusions Patients with microphthalmia often have ocular and systemic anomalies. Early assessment and preservation of vision and long-term complication management are needed.

Keywords Microphthalmos · Epidemiology · Survey · Intractable disease

S. Nishina (✉) · Y. Kobayashi · N. Azuma
Division of Ophthalmology, National Center for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan
e-mail: nishina-s@ncchd.go.jp

D. Kurosaka
Department of Ophthalmology, Iwate Medical University School of Medicine, Iwate, Japan

Y. Nishida
Department of Ophthalmology, Shiga University of Medical Science, Shiga, Japan

H. Kondo
Department of Ophthalmology, University of Occupational and Environmental Health, Japan, Fukuoka, Japan

Introduction

Microphthalmos is defined as the arrested development of all global dimensions and is often is associated with other ocular and systemic anomalies [1]. Chromosomal disorders, genetic syndromes, and environmental factors, such as maternal infection and exposure to X-rays or drugs, are reported as causes [2]. However, in most cases the precise pathogenesis is unknown although some causative genes (*SOX2* and *PAX6*) have been identified [2–4].

Previous studies conducted in the UK report that the prevalence rates of microphthalmia, anophthalmia, and typical coloboma are 10–19 per 100,000 births [4–7]. Microphthalmia is rare, and only a few disease, genetic, and epidemiologic studies and a few reports on the practical patient status have been published. The condition generally causes substantial visual impairment, but standard management and treatments have not been established.

We conducted a cross-sectional national survey to investigate the current status of patients with microphthalmia, focusing especially on ocular associations,

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complications, surgery, and vision examinations performed by ophthalmologists.

Materials and methods

A questionnaire was sent to the departments of ophthalmology in 1,151 major hospitals nationwide, all of which are authorized by the Japanese Ophthalmological Society as training institutions for physicians specializing in ophthalmology, to survey the number of patients with microphthalmia who visited their outpatient clinics between January 2008 and December 2009. Patients referred to other hospitals during this period were excluded.

The diagnostic criterion for pure microphthalmos is the presence of an eye with two-thirds the normal ocular volume, i.e., 0.87 below the normal axial length [1]. The Japanese criteria were established by Majima [8], based on the average axial length for each age group of Japanese patients. The clinical definition can be determined by a substantial size difference between the two eyes. Axial lengths of <21 mm in adults and <19 mm in 1-year-old children, i.e., two standard deviations below normal, are used. Corneal diameters of <10 mm in adults and <9 mm in infants are used for a simple diagnosis [9]. In our survey, either Majima's criteria for pure microphthalmos or the clinical definition for complicated microphthalmos was applied.

The questionnaire asked for either the numbers of patients or the number of eyes and was divided into two sheets. The first sheet comprised questions on the number of cases, the number of cases operated on, whether the condition was unilateral or bilateral, gender, age, family history; the second sheet consisted of questions about the number of associated ocular anomalies and complications, surgical treatment, associated systemic diseases, vision and management with glasses, low vision aid, and the use of a prosthetic shell.

A retrospective quantitative registry of microphthalmia was compiled from the responses from 454 hospitals (39.4%). The data from 1,254 microphthalmic eyes of 851 cases in total were collected from the first sheet, but as some hospitals did not complete the second sheet, only data from 1,069 eyes of 722 cases were collected from the second sheet. Of the data collected for these 1,069 eyes, data on the vision of 56 eyes (5.2%) were incomplete. Thus, data from 1,013 eyes were analyzed for vision.

We surveyed the number of patients managed in Japanese hospitals and analyzed the associated ocular anomalies and complications, surgical treatment, systemic diseases, vision and ophthalmic management.

Results

Of the 851 cases [396 (46.5%) male, 455 (53.5%) female] of microphthalmia reported on the first sheet, 444 (52%) were unilateral and 405 (48%) were bilateral (for two cases no information on unilateralism or bilateralism was reported). In terms of age distribution, 50% of the patients were 0–9 years and 16% were 10–19 years; between ages 20 and 79 years, the prevalence remained relatively constant, ranging between 4.3 and 6.8% (Fig. 1). Family histories were positive in 61 cases (7.2%), of which 25 cases (41%) of autosomal dominant inheritance, three cases of X-linked recessive inheritance, and one case of autosomal recessive inheritance were identified; the other 32 cases were undetermined.

The data from the 1,069 microphthalmic eyes of 722 cases retrieved from the second sheet were compiled and analyzed for associated ocular anomalies and complications, surgical treatment, associated systemic diseases, and management with glasses, low vision aids, and prosthetic shells. The ocular abnormalities and complications associated with microphthalmia are shown in Fig. 2. The identified ocular findings were nanophthalmos, coloboma (choroid, retina, lens, iris), vitreoretinal malformation (retinal dysplasia, retinal fold, persistent fetal vasculature, etc.), anophthalmos/extreme microphthalmos, anterior segment dysgenesis (Peters' anomaly, aniridia), and optic

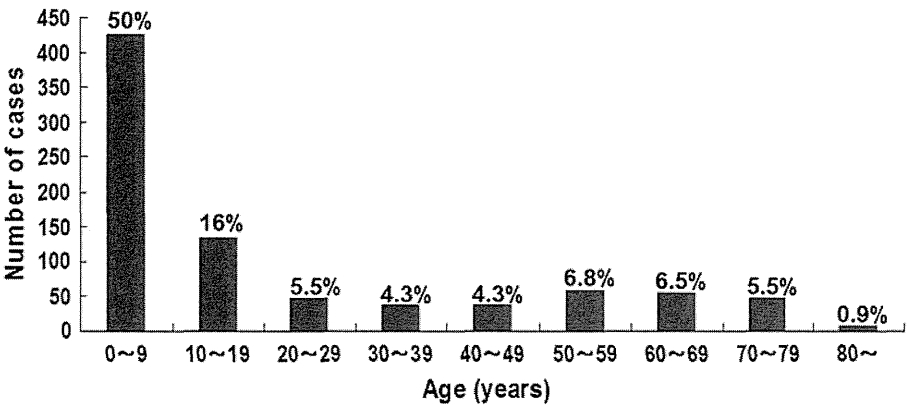

Fig. 1 Ages of patients with microphthalmia managed in the surveyed hospitals. The rate is given for each age group ($N = 851$ cases)

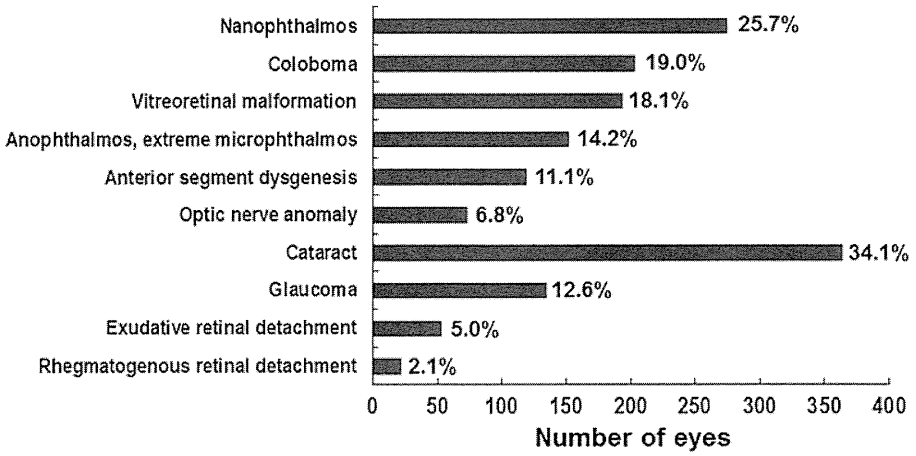

Fig. 2 Ocular abnormalities and complications associated with microphthalmia. The rate of each associated anomaly or complication is given ($N = 1{,}069$ eyes)

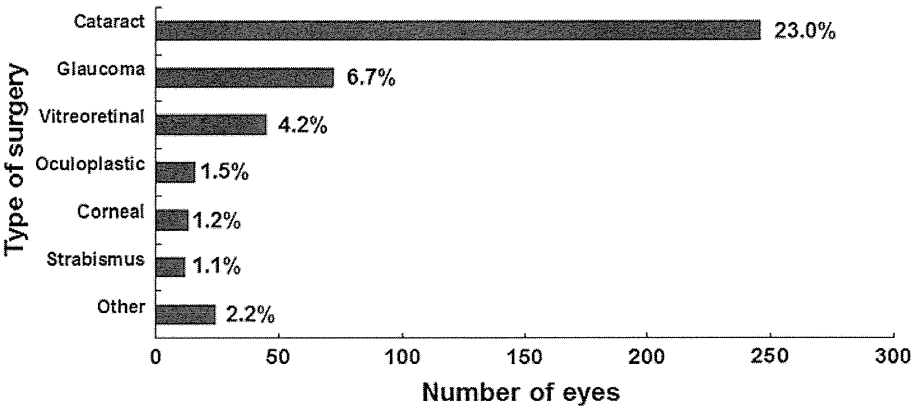

Fig. 3 Surgical treatments for ocular complications in microphthalmia. The rate of each surgical procedure is given ($N = 1{,}069$ eyes)

nerve anomaly (disc anomaly, optic nerve hypoplasia). The most frequent ocular complications were cataracts in 34.1%, followed by glaucoma and exudative or rhegmatogenous retinal detachment.

Surgery had been performed in 182 (21.4%) of the 851 cases; the surgical procedures for ocular complications are shown in Fig. 3. The procedures performed the most often were cataract extraction in 246 eyes (23.0%) of 1,069 eyes, followed by glaucoma surgery and vitreoretinal surgery.

Systemic diseases were present in 224 patients (31%) of 722 cases of microphthalmia, with 92 cases (12.7%) of developmental cerebral anomalies and mental deficiency, 68 cases (9.4%) of multiple anomalies and genetic syndromes, 26 cases (3.6%) of chromosomal disorders, and 38 cases (5.3%) of others.

The distribution of vision in microphthalmia is shown in Fig. 4. The data from 1,013 microphthalmic eyes were analyzed for vision. The visual acuity (VA) in microphthalmos was <0.02 in 348 eyes (34.4%), <0.1 but not <0.02 in 116 eyes (11.4%), <0.3 but not <0.1 in 93 eyes (9.2%), not <0.3 in 157 eyes (15.5%), unmeasurable with poor visual performance in 241 eyes (23.8%), and good visual performance in 58 eyes (5.7%).

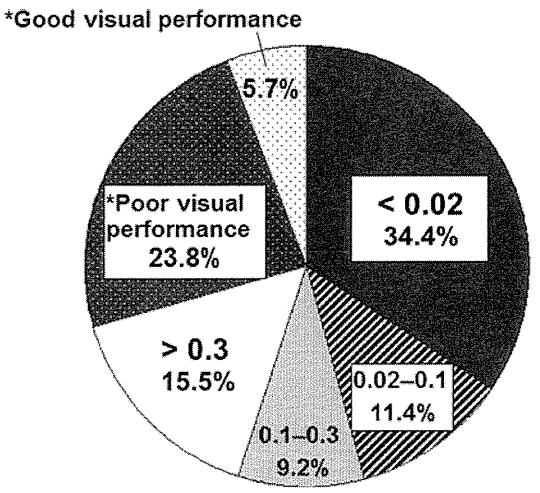

Fig. 4 Visual acuity (VA) in microphthalmos. *Asterisk* VA not measured due to young age or mental retardation. The rate of each VA group is given ($N = 1{,}013$ eyes)

Glasses and low vision aids were used in 156 cases (21.6%) of 722 cases, while prosthetic shells were applied in 211 eyes (19.7%) of 1,069 eyes.

Discussion

This is the first national survey that reports the epidemiologic aspects and current status of patients with microphthalmia in Japan. It is also the largest survey conducted by ophthalmologists of patients with microphthalmia who present at a hospital. The results based on cross-sectional surveys of patients' hospital visits may be considerably biased and may not be comparable with those of previous epidemiologic studies in other countries. However, the results of this survey showing the precise ocular associations, complications, types of surgeries, and vision, may be useful for future ocular management and investigation.

Approximately one-half of the patients in this survey who presented to a hospital were children under the age of 10 years, indicating that diagnosis and treatment of microphthalmia during the period of visual development are both needed and common practice in Japan. In addition, continuous management of low vision and ocular complications is required in order to maintain proper vision throughout life. Among the responders in this study, the distribution of microphthalmia was evenly divided between men and women and between unilateral cases and bilateral cases. Previous studies also report no biased association between microphthalmia and gender; however, those on laterality are mixed, with bilateral being more common in some studies and unilateral cases being more common in others [10]. Kallen et al. [11] reported that among their patient population, >70% of microphthalmia cases were bilateral and associated with chromosomal disorders, 53–60% were either associated or not associated with other malformations, but only 27% were cases of isolated microphthalmia. Microphthalmos associated with systemic diseases, nanophthalmos, colobomatous microphthalmos, and some cases of complicated microphthalmos often develop bilaterally and need more medical management for low vision and periodic follow-up. However, the current survey indicated that unilateral cases also require ophthalmic treatment and management.

The family histories were positive in 7.2% of cases; however, most cases have not been investigated for genetic etiology. To clarify the pathogenesis of various microphthalmia and develop useful treatments, effective genetic screening should be performed.

The current patient population had varying kinds and degrees of ocular-associated anomalies; among these, posterior segment dysgenesis, including coloboma and vitreoretinal malformations, was seen frequently. Thus, early morphologic and electrophysiologic evaluation of the posterior segment may be required to assess the visual potential and indications for surgical, optical, and amblyopia treatment or for a cosmetic shell.

The rates of developing cataracts, glaucoma, and retinal detachments were extremely high among the young patients. These ocular complications were major indications for surgical intervention, although the prognoses were generally poor [12]. Patients with microphthalmia require lifelong management for early prevention and detection of these complications. A less invasive surgical procedure for microphthalmia should be developed [13–19].

Various systemic anomalies are frequently associated with microphthalmia, indicating that initial assessment and continuous management by pediatricians are essential. Although 31% of the cases in our survey were microphthalmia associated with systemic disease, analysis of a population-based birth defects registry in Hawaii from 1986 to 2001 revealed that only 5% of the 96 cases had either isolated anophthalmia or microphthalmia, whereas 25% had confirmed chromosomal abnormalities, such as trisomy 18 and 13, and others had malformation syndromes, limb and musculoskeletal system defects, and cardiac and circulatory system defects [10]. Our survey included more unilaterally isolated cases, probably because ophthalmologists conducted the survey and provided detailed descriptions of the ocular status of the patients who presented to the hospitals.

Overall useful vision and good visual performance >0.1 were obtained in about 30% of microphthalmia cases, whereas about 34% of microphthalmia patients were blind (VA <0.02). However, glasses and low vision aids were used in around 22% of the cases, while prosthetic shells were used in about 20% of eyes. The visual prognosis of microphthalmos depends largely on the difference between the two eyes. The chances of obtaining good VA are limited in cases of severe unilateral microphthalmos, where orbital growth may be retarded and facial deformity may develop. Early socket expansion and wearing of a prosthetic shell are important for cosmetic treatment in anophthalmos and extreme microphthalmos [20]. However, microphthalmos with visual potential should be assessed early and glasses prescribed to maximize the VA.

In summary, our analysis of the survey data revealed that patients with microphthalmia have various ocular and systemic anomalies and that the rates of ocular complications are high in young patients. Early assessment, preservation of vision, and long-term management of complications are needed for these patients.

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Two Novel Mutations in the *EYS* Gene Are Possible Major Causes of Autosomal Recessive Retinitis Pigmentosa in the Japanese Population

Katsuhiro Hosono[1]*, Chie Ishigami[2], Masayo Takahashi[2], Dong Ho Park[3], Yasuhiko Hirami[4], Hiroshi Nakanishi[5], Shinji Ueno[6], Tadashi Yokoi[7], Akiko Hikoya[1], Taichi Fujita[1], Yang Zhao[1,8], Sachiko Nishina[7], Jae Pil Shin[3], In Taek Kim[3], Shuichi Yamamoto[9], Noriyuki Azuma[7], Hiroko Terasaki[6], Miho Sato[1], Mineo Kondo[6], Shinsei Minoshima[8], Yoshihiro Hotta[1]

1 Department of Ophthalmology, Hamamatsu University School of Medicine, Hamamatsu, Japan, **2** Laboratory for Retinal Regeneration, RIKEN Center for Developmental Biology, Kobe, Japan, **3** Department of Ophthalmology, Kyungpook National University Hospital, Daegu, Korea, **4** Department of Ophthalmology, Institute of Biomedical Research and Innovation Hospital, Kobe, Japan, **5** Department of Otolaryngology, Hamamatsu University School of Medicine, Hamamatsu, Japan, **6** Department of Ophthalmology, Nagoya University Graduate School of Medicine, Nagoya, Japan, **7** Department of Ophthalmology and Laboratory of Cell Biology, National Center for Child Health and Development, Tokyo, Japan, **8** Department of Photomedical Genomics, Basic Medical Photonics Laboratory, Medical Photonics Research Center, Hamamatsu University School of Medicine, Hamamatsu, Japan, **9** Department of Ophthalmology and Visual Science, Chiba University Graduate School of Medicine, Chiba, Japan

Abstract

Retinitis pigmentosa (RP) is a highly heterogeneous genetic disease including autosomal recessive (ar), autosomal dominant (ad), and X-linked inheritance. Recently, arRP has been associated with mutations in *EYS* (Eyes shut homolog), which is a major causative gene for this disease. This study was conducted to determine the spectrum and frequency of *EYS* mutations in 100 Japanese arRP patients. To determine the prevalence of *EYS* mutations, all *EYS* exons were screened for mutations by polymerase chain reaction amplification, and sequence analysis was performed. We detected 67 sequence alterations in *EYS*, of which 21 were novel. Of these, 7 were very likely pathogenic mutations, 6 were possible pathogenic mutations, and 54 were predicted non-pathogenic sequence alterations. The minimum observed prevalence of distinct *EYS* mutations in our study was 18% (18/100, comprising 9 patients with 2 very likely pathogenic mutations and the remaining 9 with only one such mutation). Among these mutations, 2 novel truncating mutations, c.4957_4958insA (p.S1653KfsX2) and c.8868C>A (p.Y2956X), were identified in 16 patients and accounted for 57.1% (20/35 alleles) of the mutated alleles. Although these 2 truncating mutations were not detected in Japanese patients with adRP or Leber's congenital amaurosis, we detected them in Korean arRP patients. Similar to Japanese arRP results, the c.4957_4958insA mutation was more frequently detected than the c.8868C>A mutation. The 18% estimated prevalence of very likely pathogenic mutations in our study suggests a major involvement of *EYS* in the pathogenesis of arRP in the Japanese population. Mutation spectrum of *EYS* in 100 Japanese patients, including 13 distinct very likely and possible pathogenic mutations, was largely different from the previously reported spectrum in patients from non-Asian populations. Screening for c.4957_4958insA and c.8868C>A mutations in the *EYS* gene may therefore be very effective for the genetic testing and counseling of RP patients in Japan.

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* E-mail: hosono@hama-med.ac.jp

Introduction

Retinitis pigmentosa (RP [MIM 268000]) is a highly heterogeneous genetic disease characterized by night blindness and visual field constriction leading to severe visual impairment. The disease appears with different modes of inheritance including autosomal recessive (ar), autosomal dominant (ad), and X-linked, and currently over half of cases are isolated in Japan.

To date, 53 causative genes and 7 loci of RP have been identified (http://www.sph.uth.tmc.edu/Retnet/), including the eyes shut homolog (*EYS*) gene encoding an ortholog of *Drosophila* spacemaker (spam), a protein essential for photoreceptor morphology. *EYS* spans over 2 Mb, making it one of the largest known genes expressed in the human eye [1,2]. *EYS* gene mutations, primarily truncating and some missense mutations, have been detected in arRP families of different ancestral origin and have reported to account for 5–16% of arRP [3–6]. Most gene mutations (e.g., *RHO*, *PRPH2*, *PRPF31*, *RP1*, and *IMPDH1*) have been found in Japanese patients with adRP, with few reports describing mutations in arRP [7,8]. Therefore, the genes causing arRP in most Japanese families have yet to be identified.

In this study, we screened all *EYS* gene exons in 100 unrelated Japanese RP patients. We found 2 novel truncating *EYS* gene mutations that were surprisingly related to 16% of Japanese arRP patients, but were not detected in Japanese patients with either adRP or Leber's congenital amaurosis (LCA [MIM204000], the earliest onset and most severe form of hereditary retinal dystrophy with several clinical features overlapping with those of RP). Additionally, these mutations were also detected in 9% of Korean arRP patients.

Methods

Patients and clinical evaluation

We screened all *EYS* gene exons in 100 unrelated Japanese RP patients with no systemic manifestations, excluding families with obvious autosomal dominant inheritance. Some pedigrees showed a pattern compatible with the recessive mode of inheritance; the other patients were considered isolated cases. In addition, 200 unrelated and non-RP Japanese individuals were screened as controls to evaluate the frequency of the mutations found in the patient samples. We also screened a part of *EYS* gene exons 26 and 44 in 19 unrelated Japanese adRP patients, 28 unrelated Japanese LCA patients, and 32 unrelated Korean arRP patients. The 19 Japanese adRP patients had already been screened for some principal adRP-causing genes, but the pathogenic mutations have not yet been detected. Examples of the screening list for adRP-causing genes and targeted exons include exon 3 and 4 in *RP1*; exon 1, 2, 3, 4, and 5 in *RHO*; exon 1, 2, and 3 in *PRPH2*; exon 2, 3, and 4 in *CRX*; exon 11 in *PRPF3*; exon 10, 11, and 12 in *IMPDH1*; exon 2 in *NRL*; exon 43 in *PRPF8*; exon 1 and 2 in *ROM1*; exon 5 and 6 in *RP9*; exon 2, 3, 5, 6, 7, 8, 11, and 12 in *PRPF31*; exon 11 and 15 in *SEMA4A*; exon 1 in *CA4*; exon 3 in *GUCA1B*; exon 3 in *SP4*; and exon 3 in *TOPORS*.

Japanese RP patients were examined either at the Department of Ophthalmology, Hamamatsu University Hospital in Hamamatsu (by YH), Department of Ophthalmology, Kobe City Medical Center General Hospital in Kobe (by MT), or Department of Ophthalmology, Nagoya University Hospital in Nagoya (by MK). Patients' origin varied widely, from the Tokyo to Osaka areas in Japan. Japanese LCA patients were examined at the Department of Ophthalmology and Laboratory of Cell Biology, National Center for Child Health and Development in Tokyo (by NA). LCA patients' origin varied widely, from all over Japan except the Okinawa islands. Meanwhile, Korean RP patients were examined at the Department of Ophthalmology, Kyungpook National University Hospital in Daegu (by ITK). The Korean patients' origin varied widely, from Daegu to Yeongju and Pohang areas in Gyeongsangbuk-do, Korea. A full ophthalmic examination was performed. Clinical diagnosis for RP was based on visual field, fundus examination, and electroretinogram findings, and clinical diagnosis for LCA was based on fundus examination and electroretinogram findings.

Ethics statements

This study was approved by the Institutional Review Board for Human Genetic and Genome Research at the 6 participating institutions (Hamamatsu University School of Medicine, RIKEN Center for Developmental Biology, Nagoya University Graduate School of Medicine, National Center for Child Health and Development, Chiba University Graduate School of Medicine, and Kyungpook National University Hospital), and its procedures conformed to the tenets of the Declaration of Helsinki. Written informed consent was obtained from all participants before molecular genetic studies.

Mutation analysis

Genomic DNA in Japanese samples was extracted from the peripheral lymphocytes using standard procedures. In Korean samples, whole blood samples were collected on FTA cards (GE Healthcare). Blood samples were spotted onto the cards and air-dried for 1 h at room temperature. For polymerase chain reaction (PCR) amplification, a 1.2-mm disk was punched from a dried blood spot using a Harris micro-punch tool (GE Healthcare) and processed according to the manufacturer's instructions. PCR was performed using the KOD -Plus- ver. 2 PCR kit (Toyobo) with the primer sets described in Table S1 for 35 cycles of 98°C for 10 s, 60°C for 30 s, and 68°C for 1 min in an automated thermal cycler (GeneAmp PCR System 9700; Applied Biosystems). PCR products were purified with Wizard SV Gel and PCR Clean-up System (Promega) or treated with Exonuclease I and Antarctic Phosphatase (New England Biolabs). Direct sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI3100 autosequencer (Applied Biosystems). For Japanese arRP patients, all 44 exons, including 3 non-coding exons (exons 1–3) that cover the 5′ untranslated region and 41 coding exons (exons 4–44), were analyzed in both sense and antisense directions. For Japanese adRP and LCA patients, and Korean arRP patients, parts of exons 26 and 44 were analyzed (Table S1).

Assessment of pathogenicity

A sequence variant was considered pathogenic if it represented a truncating mutation (nonsense or frameshift), large-scale deletion mutation, or missense mutation affecting a conserved amino acid residue and did not appear in control samples (number of alleles studied ≤400) and/or in a public SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). Particularly, missense mutations were considered pathogenic if found together with a second variant, especially if it was truncating. As reference data, we employed 4 computational algorithms to evaluate the pathogenicity of missense mutations: SIFT (http://sift.jcvi.org/www/SIFT_seq_submit2.html), PolyPhen2 (http://genetics.bwh.harvard.edu/pph2/), PMut (http://mmb.pcb.ub.es/PMut/), and SNAP (http://rostlab.org/services/snap/).

Results

Mutation analysis

Mutation analysis of *EYS* in 100 unrelated Japanese patients revealed 7 very likely pathogenic mutations in 18 patients (18%). Of these 18 patients, a second mutant allele could not be detected in 9 patients. The very likely pathogenic mutations consisted of 3 truncating mutations, 1 deletion mutation, 2 missense mutations, and 1 previously described mutation (Fig. 1, Table 1, and Table 2). In addition, we also identified 6 possible pathogenic mutations in 8 separate patients (Table 1 and Table 2).

A novel truncating insertion, c.4957_4958insA, was detected in 12 patients and accounted for 15 of the 35 mutated alleles detected (42.9%) (Table 1 and Table 2). Three patients were homozygous for the c.4957_4958insA mutation, and the other 9 patients were heterozygous. Of the latter, 3 patients showed the second mutation while 6 did not. This insertion creates a frameshift mutation that predicts a premature stop at codon 1654 (p.S1653KfsX2). A novel truncating nonsense mutation c.8868C>A (p.Y2956X) was identified in 4 patients and accounted for 5 of the 35 mutated alleles detected (14.3%). Thus, these 2 novel truncating mutations were identified in 16 separate patients, resulting in a very high frequency of the 2 mutations in Japanese arRP patients.

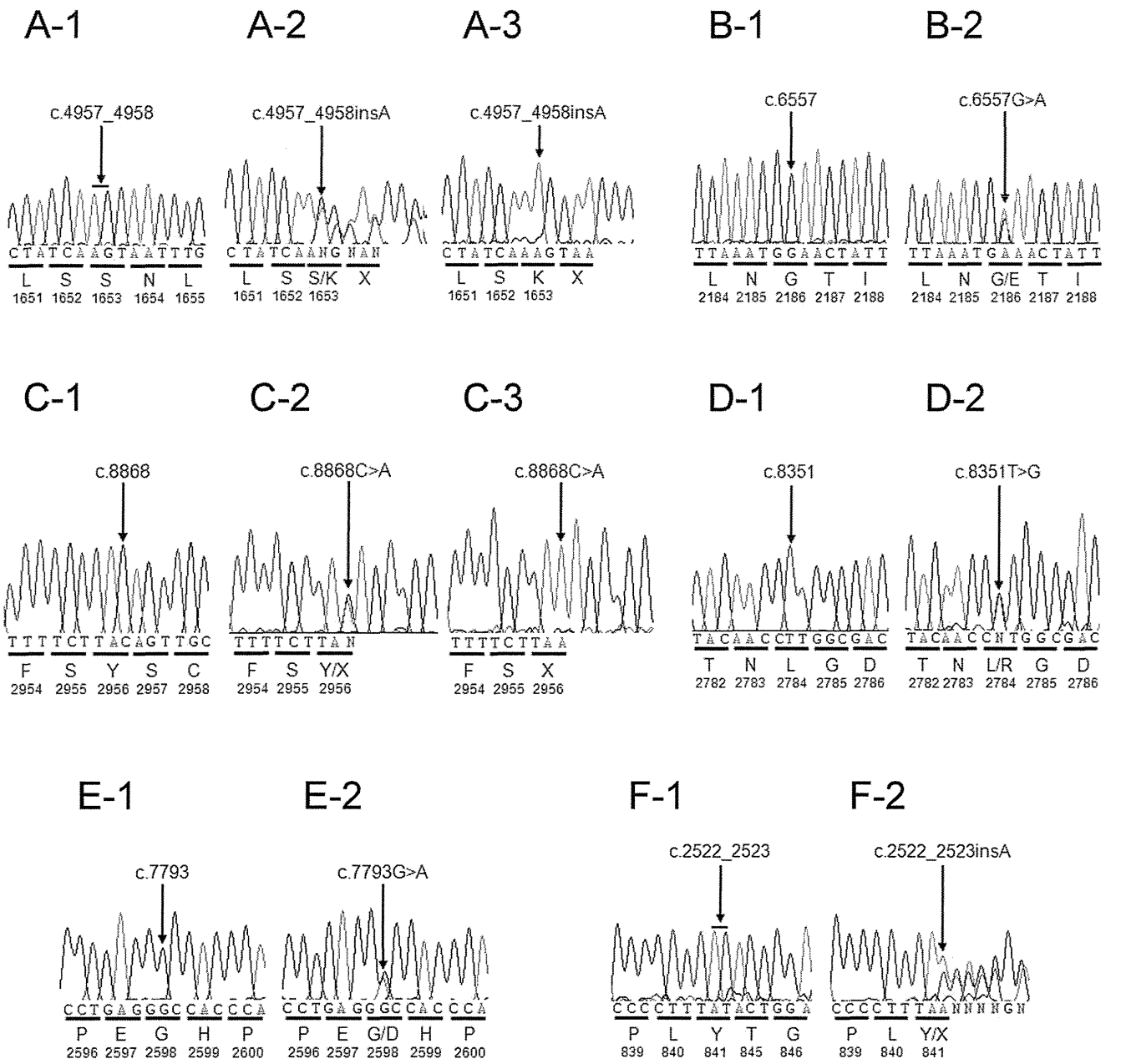

Figure 1. Electropherograms of the 6 likely pathogenic *EYS* mutations. Partial sequence of the *EYS* gene showing the normal control sequences (A-1 through F-1), heterozygous mutation sequences (A-2 through F-2), and homozygous mutation sequences (A-3 and C-3). Deduced amino acids are indicated under the sequence trace. The mutation location is indicated either by an arrow (for a nucleotide change) or a horizontal line (to show 2 nucleotides between which the insertion occurred). (A) c.4957_4958insA; p.S1653KfsX2 (Exon 26), (B) c.6557G>A; p.G2186E (Exon 32), (C) c.8868C>A; p.Y2956X (Exon 44), (D) c.8351T>G; p.L2784R (Exon 44), (E) c.7793G>A; p.G2598D (Exon 40), (F) c.2522_2523insA; p.Y841X (Exon 16). doi:10.1371/journal.pone.0031036.g001

Families with very likely pathogenic mutations and both alleles affected

Nine of the 18 patients bearing very likely pathogenic mutations appeared to have both alleles affected, suggesting that they received one mutated allele from each unaffected parent (Table 1 and Table 2). In 4 patients (RP3H, RP48K, RP56K, and RP81K), segregation analysis was performed, and the 2 pathogenic alleles were considered to be on different chromosomes (Fig. 2).

1. In RP3H, proband (II-6) was homozygous for c.4957_4958insA. The mutation co-segregated with the phenotype: the unaffected brother (II-4) demonstrated wild-type alleles, while the affected brother (II-5) was homozygous for the mutation.
2. In RP48K, proband (II-1) was homozygous for c.4957_4958insA. The unaffected brother (II-2) was heterozygous for the mutation.
3. In RP56K, proband (II-1) was compound heterozygous for c.4957_4958insA and missense mutation c.8351T>G (p.L2784R). The mutation co-segregated with the phenotype: the affected brother (II-2) also showed both mutations, while the unaffected brother (II-3) was heterozygous for c.4957_4958insA.

Table 1. Mutation spectrum of the *EYS* gene in Japanese families.

Family ID	Nucleotide change	Predicted effect	Domain[a]	Location in gene	Type of change	Reference
Families with very likely pathogenic mutations and both alleles affected						
RP3H[b]	c.4957_4958insA/ c.4957_4958insA	p.S1653KfsX2/ p.S1653KfsX2	Close to coiled-coil/ Close to coiled-coil	Exon 26/Exon 26	Homozygous	This study
RP48K[b]	c.4957_4958insA/ c.4957_4958insA	p.S1653KfsX2/ p.S1653KfsX2	Close to coiled-coil/ Close to coiled-coil	Exon 26/Exon 26	Homozygous	This study
RP54K	c.4957_4958insA/ c.4957_4958insA	p.S1653KfsX2/ p.S1653KfsX2	Close to coiled-coil/ Close to coiled-coil	Exon 26/Exon 26	Homozygous	This study
RP44K	c.4957_4958insA/ c.6557G>A	p.S1653KfsX2/ p.G2186E	Close to coiled-coil/ Laminin G	Exon 26/Exon 32	Heterozygous/ Heterozygous	This study/Abd El-Aziz et al., 2010; Littink et al., 2010; This study
RP56K[b]	c.4957_4958insA/ c.8351T>G	p.S1653KfsX2/ p.L2784R	Close to coiled-coil/ Laminin G	Exon 26/Exon 44	Compound Heterozygous	This study
RP87N	c.4957_4958insA/ c.7793G>A	p.S1653KfsX2/ p.G2598D	Close to coiled-coil/ Close to Laminin G	Exon 26/Exon 40	Heterozygous/ Heterozygous	This study
RP81K[b]	c.2522_2523insA/ c.6557G>A	p.Y841X/p.G2186E	EGF/Laminin G	Exon 16/Exon 32	Compound Heterozygous	This study/Abd El-Aziz et al., 2010; Littink et al., 2010; This study
RP21H	deletion exon32/ deletion exon32	p.D2142_S2191delinsG/ p.D2142_S2191delinsG	Laminin G/Laminin G	Exon 32/Exon 32	Homozygous	This study
RP35K	c.8868C>A/c.8868C>A	p.Y2956X/p.Y2956X	EGF/EGF	Exon 44/Exon 44	Homozygous	This study
Families with single very likely pathogenic mutations						
RP1H	c.4957_4958insA	p.S1653KfsX2	Close to coiled-coil	Exon 26	Heterozygous	This study
RP6H	c.4957_4958insA	p.S1653KfsX2	Close to coiled-coil	Exon 26	Heterozygous	This study
RP12H	c.4957_4958insA	p.S1653KfsX2	Close to coiled-coil	Exon 26	Heterozygous	This study
RP51K	c.4957_4958insA	p.S1653KfsX2	Close to coiled-coil	Exon 26	Heterozygous	This study
RP96H	c.4957_4958insA	p.S1653KfsX2	Close to coiled-coil	Exon 26	Heterozygous	This study
RP100N	c.4957_4958insA	p.S1653KfsX2	Close to coiled-coil	Exon 26	Heterozygous	This study
RP8H	c.8868C>A	p.Y2956X	EGF	Exon 44	Heterozygous	This study
RP25H	c.8868C>A	p.Y2956X	EGF	Exon 44	Heterozygous	This study
RP80K[b]	c.8868C>A	p.Y2956X	EGF	Exon 44	Heterozygous	This study
Families with single possible pathogenic mutations						
RP4H	c.9272T>C	p.I3091T	Laminin G	Exon 44	Heterozygous	This study
RP9H	c.8875C>A	p.L2959M	EGF	Exon 44	Heterozygous	This study
RP49K	c.9272T>C	p.I3091T	Laminin G	Exon 44	Heterozygous	This study
RP53K	c.5884A>G	p.T1962A	Laminin G	Exon 28	Heterozygous	This study
RP55K	c.9272T>C	p.I3091T	Laminin G	Exon 44	Heterozygous	This study
RP74K	c.5404C>T	p.L1802F	Close to Laminin G	Exon 26	Heterozygous	This study
RP79K	c.77G>A	p.R26Q	Close to signal peptide cleavage site	Exon 4	Heterozygous	This study
RP83K	c.2923T>C	p.C975R	EGF	Exon 19	Heterozygous	This study

Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence FM209056, according to the nomenclature recommended by the Human Genome Variation Society (www.hgvs.org/mutnomen). The initiation codon is codon 1. None of these 13 mutations were found in the Japanese controls.

[a]EYS has a signal peptide, a putative coiled-coil, 29 EGF, and 5 Laminin G domains. See Fig. 3.

[b]Segregation analysis has been performed. See Fig. 2.

In RP56K and RP81K, 2 pathogenic alleles were considered to be on different chromosomes (compound heterozygous). See Fig. 2.

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4. In RP81K, proband (II-5) was compound heterozygous for truncating insertion c.2522_2523insA (p.Y841X) and missense mutation c.6557G>A (p.G2186E). This insertion results in premature termination of the encoded protein at codon 841 (p.Y841X). Missense mutation c.6557G>A has been previously reported as disease causing in one Korean/American and one Chinese patient [3,6]. The unaffected mother (I-2) was heterozygous for c.2522_2523insA, while the unaffected sister (II-6) was heterozygous for c.6557G>A.

For the other patients, segregation analysis could not be performed due to difficulties in collecting samples from the families of patients (Table 1). RP54K and RP35K were homozygous for truncating mutation c.4957_4958insA and c.8868C>A, respectively. RP21H was homozygous for deletion in exon 32, an in-frame deletion that results in the replacement of amino acids from D2142 to S2191 with G2142 (p.D2142_S2191delinsG) and disrupts the second laminin G domain (Fig. 3). RP44K and RP87N were heterozygous for truncating and missense mutations, c.4957_4958insA/c.6657G>A (p.G2186E) and

Table 2. Summary of the very likely and possible pathogenic mutations identified in 100 Japanese arRP patients.

							Allele frequency					Computational prediction[c]			
		Nucleotide change	Predicted effect	Location in gene	Domain[a]	Conservation in hu/o/m/ho/d/op/p/c/z/dr[b]	Control	Patient	Family ID	Reference	Species	SIFT	PolyPhen2 (HumDiv)	PMut	SNAP
Very likely pathogenic mutations	**Insertion**	c.2522_2523insA	p.Y841X	Exon 16	EGF	not applicable	0/400	1/200	RP81K	This study	Japanese				
		c.4957_4958insA	p.S1653KfsX2	Exon 26	Close to coiled-coil	not applicable	0/400	15/200	RP1H, RP3H, RP6H, RP12H, RP48K, RP51K, RP54K, RP44K, RP56K, RP87N, RP96H, RP100N	This study	Japanese				
	Nonsense	c.8868C>A	p.Y2956X	Exon 44	EGF	not applicable	0/400	5/200	RP8H, RP25H, RP35K, RP80K	This study	Japanese				
	Deletion	Deletion exon 32	p.D2142_S2191delinsG	Exon 32	Laminin G	not applicable	0/200[d]	2/200	RP21H	This study	Japanese				
	Missense	c.6557G>A	p.G2186E	Exon 32	Laminin G	G/G/G/G/G/-/-/-/-/-	0/400	2/200	RP44K, RP81K	Abd El-Aziz et al., 2010; Littink et al., 2010; This study	Chinese, South Korean/American, Japanese		Probably damaging	Pathological	Non-neutral
		c.7793G>A	p.G2598D	Exon 40	Close to Laminin G	G/G/G/-/-/-/-/G/I/T	0/400	1/200	RP87N	This study	Japanese		Probably damaging		Non-neutral
		c.8351T>G	p.L2784R	Exon 44	Laminin G	L/L/L/L/L/L/L/L/L/G	0/400	1/200	RP56K	This study	Japanese		Probably damaging		Non-neutral
Possible pathogenic mutations	**Missense**	c.77G>A	p.R26Q	Exon 4	Close to signal peptide cleavage site	R/R/R/K/K/-/-/-/-/-	0/400	1/200	RP79K	This study	Japanese	Affected protein function		Pathological	
		c.2923T>C	p.C975R	Exon 19	EGF	C/C/C/-/-/-/-/-/-/-	0/400	1/200	RP83K	This study	Japanese		Possibly damaging	Pathological	Non-neutral
		c.5404C>T	p.L1802F	Exon 26	Close to Laminin G	L/L/L/-/-/-/-/-/-/-	0/400	1/200	RP74K	This study	Japanese		Possibly damaging		
		c.5884A>G	p.T1962A	Exon 28	Laminin G	T/T/T/T/-/-/-/-/-/-	0/400	1/200	RP53K	This study	Japanese		Possibly damaging		
		c.8875C>A	p.L2959M	Exon 44	EGF	L/L/L/L/L/L/A/V/-/S	0/400	1/200	RP9H	This study	Japanese		Possibly damaging		
		c.9272T>C	p.I3091T	Exon 44	Laminin G	I/I/I/I/I/I/I/I/I/L	0/400	3/200	RP4H, RP49K, RP55K	This study	Japanese	Affected protein function	Probably damaging		

[a]EYS contains a signal peptide, a putative coiled-coil, 29 EGF, and 5 laminin G domains. See Fig. 3.

[b]hu/o/m/ho/d/op/p/c/z/dr denotes Human/Orangutan/Marmoset/Horse/Dog/Opossum/Platypus/Chicken/Zebrafish/Drosophila EYS orthologs, respectively. The hyphen (-) indicates that genomic sequence of corresponding region in the species was reported to be unknown [5].

[c]SIFT, PolyPhen2 (only the HumDiv data are shown), PMut, and SNAP were used as reference data to evaluate the pathogenicity of the missense mutations. c.77G>A, c.2923T>C, c.7793G>A, c.8351T>G, and c.9272T>C were predicted to be pathogenic by a number of different computational prediction programs. In addition, the c.6557G>A mutation, which had been previously reported as disease causing, was classified as pathogenic by the PolyPhen2, PMut, and SNAP programs.

[d]Homozygous exon 32 deletion mutation was not detected in 200 controls.

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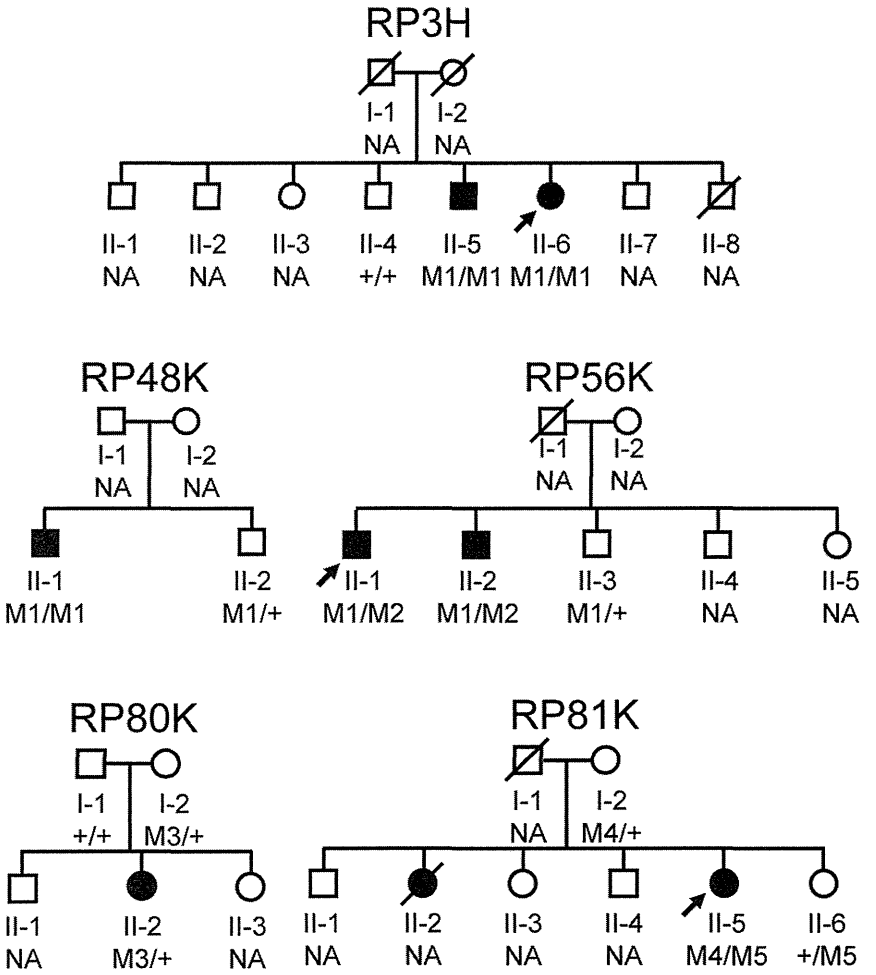

Figure 2. Pedigrees of the families that was available for mutation analysis. Below the individuals, genotypes are presented for either p.S1653KfsX2 (M1), p.L2784R (M2), p.Y2956X (M3), p.Y841X (M4), or p.G2186E (M5) detected to segregate with RP. M1/M1 represents homozygous mutation. M1/+ indicates heterozygous carriers, +/+ indicates individuals carrying 2 wild-type alleles, whereas M1/M2 represents individuals presenting both mutations as compound heterozygous. Square boxes indicate men, circles denote women, and affected individuals are pointed out by a black symbol. Slashed symbols indicate deceased individuals. The probands are indicated with an arrow. NA denotes unavailable DNA samples.
doi:10.1371/journal.pone.0031036.g002

c.4957_4958insA/c.7793T>G (p.G2598D), respectively. None of these 7 very likely pathogenic mutations were found in the Japanese controls.

Families with single novel very likely pathogenic mutations

The rest of the patients comprising the group with very likely pathogenic mutations presented only single truncating mutations (Table 1 and Table 2). RP1H, RP6H, RP12H, RP51H, RP96H, and RP100N were heterozygous for c.4957_4958insA. RP8H, RP25H, and RP80K were heterozygous for c.8868C>A. Segregation analysis was performed in patient RP80K. The unaffected father (I-1) demonstrated wild-type alleles, and the unaffected mother (I-2) was heterozygous for the mutation (Fig. 2). In RP96H, we found very likely pathogenic missense mutation c.8923T>C (p.F2975L), which was not detected in any of the 400 control alleles. However, as c.8923T>C has been described as rs79036642 in the dbSNP database, it was assigned to the group of possible non-pathogenic sequence alterations (Table 3).

Families with single novel possible pathogenic mutations

A group of patients with possible pathogenic mutations had only single missense mutations (Table 1 and Table 2). We report 6 novel missense mutations in 8 different patients (Table 1 and Table 2), none of which were identified in the 400 Japanese control alleles. All amino acid residues affected by these mutations were compared with those encoded by orthologous genes of various vertebrates (orangutan, marmoset, horse, dog, opossum, platypus, chicken, and zebrafish) and *Drosophila* and found to be highly conserved across species (Table 2). The novel missense mutation c.2923T>C (p.C975R) was predicted to be pathogenic by 3 different computational prediction programs (PolyPhen2, PMut, and SNAP) (Table 2). RP4H, RP49K, and RP55K were heterozygous for the same missense mutation c.9272T>C (p.I3091T), which was predicted to be pathogenic by SIFT and PolyPhen2 programs (Table 2). In addition, 54 possible non-pathogenic sequence alterations were found, of which 9 were previously unreported (Table 3).

Screening of the 2 truncating mutations

We focused on 2 truncating mutations, c.4957_4958insA in exon 26 and c.8868C>A in exon 44, which were identified in 16 separate Japanese arRP patients in this study. The frequency of the 2 mutations was very high in this Japanese arRP cohort. However, we did not detect the 2 mutations in 19 Japanese adRP

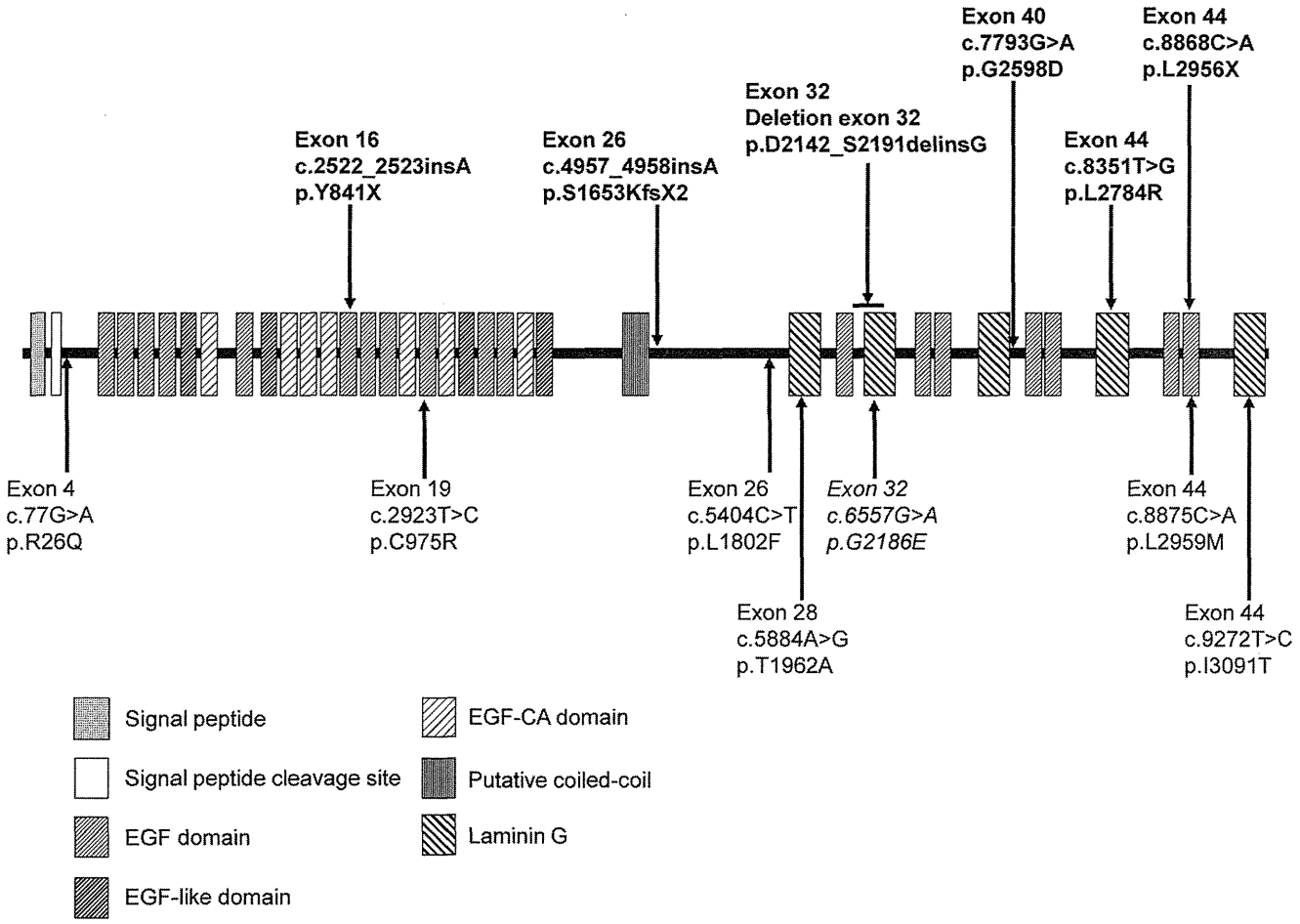

Figure 3. Predicted domain structure and distribution of identified *EYS* mutations. SMART (http://smart.embl-heidelberg.de/) and Pfam (http://pfam.sanger.ac.uk/) were used to search protein functional domains. A coiled-coil domain identified by Barragán et al. (2010) between the EGF-like domain and laminin G domain was also indicated. Novel very likely pathogenic mutations, novel possible pathogenic mutations, and a previously described mutation are shown in bold, normal, and italic type, respectively. Six out of 9 missense mutations were found in the EGF or laminin G domains. Furthermore, 7 were located in the latter half of the protein between the putative coiled-coil region and C-terminus.
doi:10.1371/journal.pone.0031036.g003

patients and 28 LCA patients who were recruited and screened to evaluate the frequency of the mutations. We also recruited 32 unrelated Korean arRP patients and screened for the 2 *EYS* gene mutations. The c.4957_4958insA mutation was detected in 2 patients and accounted for 3 of 64 Korean patient alleles (4.7%). One patient was homozygous and the other was heterozygous. The c.8868C>A mutation was identified in 1 patient and accounted for 1 of the 64 Korean patient alleles (1.6%).

Clinical findings

Nine Japanese patients with very likely pathogenic *EYS* gene mutations in both alleles, 9 Japanese patients with single very likely pathogenic changes, and a Korean patient with homozygous c.4957_4958insA mutation demonstrated classic RP with mostly night blindness as the initial symptom, followed by gradual constriction of the visual field. The fundus displayed bone spicules increasing in density with age and attenuated retinal vessels. Electroretinogram responses were not detectable, consistent with severe generalized rod-cone dysfunction. The remaining visual field determined using Goldmann kinetic perimetry with V-4 target ranged from approximately 10° to 60° of the central and inferior visual fields, respectively, in a 74-year-old woman (RP100N) to complete blindness in a 54-year-old man (RP21H). No remarkable clinical difference was observed between 9 patients with very likely pathogenic *EYS* gene mutations in both alleles and 9 patients with single very likely pathogenic changes.

Discussion

This study is the first to analyze mutations in the *EYS* gene among Japanese arRP patients. We detected 67 sequence alterations in the *EYS* gene, of which 21 were novel. Of these, 7 were very likely pathogenic mutations, 6 were possibly pathogenic mutations, and 54 were possible non-pathogenic sequence alterations (Table 1, Table 2, and Table 3).

Considering only the very likely pathogenic mutations, the minimum observed prevalence of distinct *EYS* gene mutations in our study is 18% (18/100, 9 patients with 2 very likely pathogenic mutations and 9 with only one such mutations). Additionally, if the possible pathogenic mutations are included in the prevalence estimation, prevalence increases to 26% (26/100, with 17 of 26 patients presenting single mutations). The estimated prevalence in our study may be extremely high compared with those in the previous studies [3–6]. Until recently, mutations in 34 genes have been associated with arRP (http://www.sph.uth.tmc.edu/Retnet/). The most frequently mutated gene is *USH2A*, accounting for

Table 3. Summary of the possible non-pathogenic sequence alterations in the *EYS* gene identified in this study.

Gene exon	Nucleotide change	Predicted effect	Conservation in hu/o/m/ho/d/op/p/c/z/dr[a]	Patient frequency	Control frequency	SNP ID	Reference
Exon 1	c.-500A>G			13/200		rs1490127	Abd El-Aziz et al., 2010
Exon 4	c.334G>A	p.V112I	V/I/I/I/I/I/-/-/-/E	1/200	0/192	rs112609906	
	c.359C>T	p.T120M	T/T/T/T/T/A/-/-/-/I	60/200		rs12193967	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.525_527delGGA	p.176delE	E/E/E/E/E/A/-/-/-/G	1/200	1/192		This study
Intron 5	c.863-23_863-22insTT			53/200		rs34154043	Abd El-Aziz et al., 2010
	c.863-23_863-22insTTT			44/200			This study
Exon 6	c.1005G>T	p.E335D	E/E/D/-/-/-/-/-/-/-	3/200		rs80095433	
Exon 7	c.1146T>C	p.N382N	N/N/T/-/-/-/-/-/-/-	97/200		rs974110	Audo et al., 2010; Abd El-Aziz et al., 2010
Intron 8	c.1300-3C>T			117/200		rs1936439	Audo et al., 2010; Abd El-Aziz et al., 2010
Exon 9	c.1382G>A	p.C461Y	C/C/Y/-/-/-/-/-/-/-	8/200	4/192	rs76754818	Littink et al., 2010
Intron 9	c.1599+96A>C			200/200		rs1502963	Abd El-Aziz et al., 2010
Intron 10	c.1600-38G>A			12/200		rs1502965	Abd El-Aziz et al., 2010
Exon 11	c.1712A>G	p.Q571R	Q/Q/Q/-/-/-/-/-/-/-	26/200		rs61753610	Audo I et al., 2010
Exon 12	c.1809C>T	p.V603V	V/V/V/-/-/-/-/-/-/-	178/200		rs9345601	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.1891G>A	p.G631S	G/S/E/C/C/-/-/-/-/-	178/200		rs9342464	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.1922A>T	p.E641V	E/E/E/E/E/-/-/-/-/-	18/200		rs17411795	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.1985G>T	p.R662M	R/R/R/S/S/-/-/-/-/-	8/200	3/96		This study
Intron 12	c.2023+6_2023+7insT			175/200		rs67504324	
	c.2024-14C>T			3/200		rs45628235	
Intron 15	c.2382-26C>G			106/200		rs9445437	
Exon 16	c.2490T>C	p.P830P	P/P/P/P/P/P/P/Q/P/-	2/200	1/392		This study
	c.2528G>A	p.G843E	G/G/G/G/G/G/G/G/A/G	16/200	9/192	rs74419361	
	c.2555T>C	p.L852P	L/P/P/-/S/P/S/P/-/E	106/200		rs9294631	Audo et al., 2010; Abd El-Aziz et al., 2010
Intron 18	c.2846+52_2846+53insTAAT			120/200		rs66504228	Abd El-Aziz et al., 2010
	c.2847-24C>T			178/200		rs7743515	
Exon 19	c.2980C>G	p.P994A	P/P/P/-/-/-/-/-/-/-	3/200	2/192		This study
Intron 22	c.3444-5C>T			69/200		rs9445051	Audo et al., 2010; Abd El-Aziz et al., 2010
Intron 23	c.3568+60delA			1/200			This study
Exon 25	c.3787A>G	p.I1263V	I/V/V/V/V/-/-/-/-/I	36/200		rs17404123	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.3809T>G	p.V1270G	V/V/V/V/V/-/-/-/-/P	1/200	1/192		This study
Intron 25	c.3877+17_22delAGATA			36/200			Barragán I et al., 2010
Exon 26	c.3906C>T	p.H1302H	H/H/H/H/H/-/-/-/-/S	10/200		rs12663916	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.3936A>G	p.T1312T	T/A/T/A/A/-/-/-/-/S	10/200		rs12662610	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.3973C>G	p.Q1325E	Q/E/K/K/K/-/-/-/-/S	12/200		rs12663622	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.4026C>T	p.S1342S	S/S/S/S/S/-/-/-/-/A	10/200		rs12663619	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.4081A>G	p.I1361V	I/I/T/V/V/-/-/-/-/S	12/200		rs17403955	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.4256T>C	p.L1419S	L/S/S/S/S/L/S/V/Q/V	137/200		rs624851	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.4352T>C	p.I1451T	I/T/T/K/K/-/-/-/-/T	13/200		rs62415828	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.4543C>T	p.R1515W	R/R/R/R/R/-/-/-/-/H	36/200		rs62415827	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.4549A>G	p.S1517G	S/G/D/T/T/-/-/-/-/H	36/200		rs62415826	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.4593G>A	p.E1531E	E/E/E/E/E/-/-/-/-/Q	36/200		rs62415825	Audo et al., 2010; Abd El-Aziz et al., 2010
	c.5244A>C	p.L1748F	L/L/L/L/L/-/-/-/-/F	8/200		rs57312007	Audo I et al., 2010; Littink et al., 2010
	c.5617C>G	p.L1873V	L/L/L/P/P/-/-/-/-/I	38/200		rs16895517	Audo I et al., 2010
Exon 27	c.5705A>T	p.N1902I	N/N/N/N/N/P/-/R/-/A	90/200		rs9353806	Audo et al., 2010; Abd El-Aziz et al., 2010
Intron 28	c.5928-35T>C			118/200		rs587278	Abd El-Aziz et al., 2010
Intron 29	c.6078+68A>G			81/200		rs36133910	Abd El-Aziz et al., 2010
	c.6079-4_6079-3delTC			87/200		rs35395170	Audo I et al., 2010
Intron 34	c.6834+61T>G			60/200		rs66502009	Abd El-Aziz et al., 2010

Table 3. Cont.

Gene exon	Nucleotide change	Predicted effect	Conservation in hu/o/m/ho/d/op/p/c/z/dr[a]	Patient frequency	Control frequency	SNP ID	Reference
Exon 35	c.6977G>A	p.R2326Q	R/R/R/L/L/L/L/L/I/L	95/200		rs4710457	Audo et al., 2010; Abd El-Aziz et al., 2010
Exon 37	c.7394C>G	p.T2465S	T/T/T/T/T/T/T/T/S/F	8/200	2/176		This study
Exon 39	c.7666A>T	p.S2556C	S/S/S/S/S/N/S/H/E/E	57/200		rs66462731	Audo et al., 2010; Abd El-Aziz et al., 2010; Barragán et al., 2010; Littink et al., 2010
Intron 41	c.8071+84T>G			53/200		rs4710257	Abd El-Aziz et al., 2010
Exon 44	c.8923T>C	p.F2975L	F/F/F/F/F/F/F/F/-/K	1/200	0/400	rs79036642	
	c.9300A>G	p.L3100L	L/L/L/L/L/L/L/L/V/I	4/200	2/192		This study

Fifty-four sequence alterations were identified in 100 patients. These alterations were predicted to be non-pathogenic for various reasons. Some have been reported as polymorphisms in previous reports. Newly identified alterations within the exons, except for c.334G>A and c.8923T>C, were also found in the control chromosome. The hyphen (-) indicates that genomic sequence of corresponding region in the species was reported to be unknown [5].

[a]hu/o/m/ho/d/op/p/c/z/dr denotes Human/Orangutan/Marmoset/Horse/Dog/Opossum/Platypus/Chicken/Zebrafish/Drosophila EYS orthologs, respectively.

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approximately 7% of arRP cases [9,10], whereas most other genes contribute to only 1% to 2% of arRP cases [11]. The estimated prevalence of very likely and possible pathogenic mutations of the *EYS* gene in our study was 26%, suggesting its major involvement in the pathogenesis of arRP in the Japanese population.

We found that 16% of Japanese arRP patients displayed at least one c.4957_4958insA or c.8868C>A mutation, which accounted for 57.1% (15+5/35) of the mutated alleles. Thus, these mutations seem to be frequent among Japanese arRP patients. Previous studies employing Indonesian, Pakistani, Chinese, Israeli, Spanish, French, British, Dutch, and Palestinian RP patient populations have not detected them [3–6,12–15]. Since the Japanese were divided into small semi-closed population groups among which intercommunication was quite less until the mid-20th century, obvious or latent consanguineous marriages were carried out more frequently, leading to relatively high inbreeding levels in those populations. The frequency of the c.4957_4958insA and c.8868C>A mutations may result from a founder effect like that of the 2299delG *USH2A* gene mutation, which accounts for 44% of disease alleles in Danish and Norwegian patients with Usher syndrome type II [16].

We detected 13 different very likely and possible pathogenic mutations. Three were truncating mutations and accounted for 60% (21/35) of mutated alleles. Likewise, previous studies reported that most pathogenic mutations were truncated type (nonsense, deletion, insertion, or splicing) [3–6,12–15]. Furthermore, c.6557G>A was the only mutation that was common between the Japanese and other populations. This mutation has been found in Korean/American and Chinese patients [3,6]. These results indicate that the *EYS* gene mutation spectrum among Japanese patients largely differs from that among the previously mentioned non-Asian populations. The Japanese and Korean mutation spectrum may resemble each other, but an accurate comparison could not be made, because further *EYS* gene analysis of Korean RP patients is required to clarify this possibility.

A second mutant allele could not be detected by direct sequencing in 17 of 26 patients in our study. Previous studies reported 7 of 10 [3] and 9 of 17 [5] patients with heterozygous *EYS* gene mutation, implying that this finding could be due to relatively large heterozygous deletions [15]. The second mutation in these families may also have been located within the gene regulatory elements or unknown exons including alternative splicing areas. Although rare, a single *EYS* mutation in combination with another mutation on a second gene could also explain this phenotype [3].

The c.4957_4958insA and c.8868C>A mutations were not detected in Japanese patients with adRP or with LCA. Abd El-Aziz et al. reported that *EYS* gene mutation screening did not reveal any pathogenic mutations in 95 British and Chinese adRP patients [3]. Bandah-Rosenfeld et al. reported that no mutation was found in 2 Oriental Jewish and Israeli Muslim LCA patients who had a large homozygous region harboring the *EYS* gene [12]. Although further analysis of all *EYS* gene exons is required, *EYS* gene mutations may not be detected in Japanese patients with adRP and LCA. The c.4957_4958insA and c.8868C>A mutations were also detected in Korean patients with arRP and accounted for 6.3% (4/64 alleles) of the disease alleles. Similar to Japanese arRP results, the c.4957_4958insA mutation was more frequently detected than the c.8868C>A mutation. The fact that both c.4957_4958insA and c.8868C>A mutations were also detected in Korean patients suggests the possibility that the mutations occurred in an ancient common ancestor and spread throughout East Asia.

RP is a highly heterogeneous disease, with a reported prevalence rate of 1 in 4,000–8,000 people in Japan. Given the population of Japan, approximately a 100 million, the number of patients with RP can be estimated to be 12,500–25,000. The relative frequencies of RP inheritance patterns in Japanese patients were estimated as 25.2% for autosomal recessive, 16.9% for autosomal dominant, 1.6% for X-linked, and 56.3% for simplex, indicating that most Japanese RP patients represent arRP or isolated cases [17]. Autosomal recessive and simplex cases account over 80% of RP cases in Japan (approximately 10,000–20,000 people). Our results indicate that c.4957_4958insA and c.8868C>A mutations are possibly present in 1,600–3,200 Japanese patients with RP. These 2 novel mutations will be very useful for genetic diagnosis and counseling, and analysis of the mutated proteins may be helpful in the development of effective therapies for RP in Japan and Korea.

In conclusion, mutation screening of the *EYS* gene in 100 Japanese patients revealed 13 different pathogenic mutations, confirming that the mutation spectrum in Japanese patients differs from the previously reported spectrum in patients of non-Asian populations. Among these 13 mutations, 2 truncating mutations, c.4957_4958insA and c.8868C>A, were detected in at least one mutated allele in 16% of Japanese arRP patients and may be the

most frequent mutations causing RP in the Japanese populations. Screening for c.4957_4958insA and c.8868C>A mutations in the *EYS* gene is, therefore, very effective for the genetic testing and counseling of RP patients in Japan. Further analysis is necessary to obtain a more precise mutation spectrum and to identify other frequent mutations in other East Asian populations.

Supporting Information

Table S1 PCR primer sequences for human *EYS*.
(DOC)

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Author Contributions

Conceived and designed the experiments: KH MT SY MK YH. Performed the experiments: KH CI YZ. Analyzed the data: KH CI. Contributed reagents/materials/analysis tools: MT DHP YH HN SU TY AH TF SN JPS ITK SY NA HT MS MK YH. Wrote the paper: KH SM YH.

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p53 Mutation suppresses adult neurogenesis in medaka fish (*Oryzias latipes*)

Yasuko Isoe [a], Teruhiro Okuyama [a], Yoshihito Taniguchi [b], Takeo Kubo [a], Hideaki Takeuchi [a,*]

[a] *Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan*
[b] *Department of Preventive Medicine and Public Health, School of Medicine, Keio University, 35, Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*

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ABSTRACT

Tumor suppressor *p53* negatively regulates self-renewal of neural stem cells in the adult murine brain. Here, we report that the *p53* null mutation in medaka fish (*Oryzias latipes*) suppressed neurogenesis in the telencephalon, independent of cell death. By using 5-bromo-29-deoxyuridine (BrdU) immunohistochemistry, we identified 18 proliferation zones in the brains of young medaka fish; *in situ* hybridization showed that *p53* was expressed selectively in at least 12 proliferation zones. We also compared the number of BrdU-positive cells present in the whole telencephalon of wild-type (WT) and *p53* mutant fish. Immediately after BrdU exposure, the number of BrdU-positive cells did not differ significantly between them. One week after BrdU-exposure, the BrdU-positive cells migrated from the proliferation zone, which was accompanied by an increased number in the WT brain. In contrast, no significant increase was observed in the *p53* mutant brain. Terminal deoxynucleotidyl transferase (dUTP) nick end-labeling revealed that there was no significant difference in the number of apoptotic cells in the telencephalon of *p53* mutant and WT medaka, suggesting that the decreased number of BrdU-positive cells in the mutant may be due to the suppression of proliferation rather than the enhancement of neural cell death. These results suggest that *p53* positively regulates neurogenesis via cell proliferation.

1. Introduction

In the adult brain of teleosts, most proliferating cells are observed in well-defined zones of the brain (called proliferation zones) [1]. The whole brain of teleosts, such as medaka (*Oryzias latipes*) [2], zebrafish (*Danio rerio*) [3], gymnotiform fish (*Apteronotus leptorhynchus*) [4], and three-spined stickleback (*Gasterosteus aculeatus*) [5], contains a large number of proliferation zones. Previously, we identified 17 proliferation zones (Zones A–Q) in the adult medaka brain using sexually mature fish (age, more than 3 months) and demonstrated that there is persistent cell proliferation in these brain regions in the adult brain, irrespective of sex, body color, or growth environment [2]. Further, the distribution of proliferation zones is largely conserved among some fish species [2], suggesting that this distribution in the adult teleost brain is important for the maintenance and development of the fundamental structure of fish brains [2].

To clarify the molecular basis underlying adult neurogenesis in teleost fish, we focused on medaka *p53* mutants [6]. p53 is a sequence-specific DNA-binding transcription factor that induces apoptosis or cell cycle arrest in response to genotoxic stress, thus preventing DNA mutations from transmitting to progeny cells [7]. In murine brains, the *p53* null mutation enhanced cell proliferation in the adult subventricular zone (SVZ) and, in association with their rapid differentiation, resulted in an increased number of newborn neurons and oligodendrocytes [8–11]. Here, we show the distribution of proliferating zones largely overlapped that of *p53*-expressing cells in the medaka brain. Furthermore, the medaka *p53* null mutant phenotype suggested that *p53* positively regulates neurogenesis.

2. Materials and methods

2.1. Fish

Medaka fish (*O. latipes*), Cab strain and *p53* mutants [6], were maintained in groups in plastic aquariums (12 × 13 × 19 cm). Sexually immature medaka fish (approximately 1 month after hatching; body length, 15 mm) without secondary sexual characteristics were used for immunohistochemistry and *in situ* hybridization studies.

2.2. Detection of mitotic cells in the young medaka brain

The detection of mitotic cells was performed as described previously [2]. Dividing cells were labeled with 5-bromo-29-deoxyuridine (BrdU), by exposure to water containing 1 g/L BrdU (Sigma Aldrich, Tokyo) for 4 h. BrdU-positive cells were detected by anti-BrdU immunohistochemistry. Paraffin sections (10-μm thick) were cut with a microtome (LR-85, Yamato Kohki, Tokyo). Immunostain-

* Corresponding author. Fax: +81 3 5841 4448.
E-mail address: takeuchi@biol.s.u-tokyo.ac.jp (H. Takeuchi).

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ing was performed following standard procedures. Cell nuclei were detected with DAPI staining (Invitrogen, Tokyo). BrdU-positive cells were counted as described previously [2].

2.3. In situ hybridization

In situ hybridization of tissue sections was performed as described previously [12,13]. The *p53* cDNA fragment was amplified with forward primer 5′-**TGTTACATTTTATAGCTGTGGAGCA**-3′ and reverse primer 5′-**TTGGGCTGAAAACAGCACAACCATAGTT**-3′ using cDNA clone number orbr44c15 (Medaka National BioResource Project [14]) as a template. The digoxigenin (DIG)-labeled riboprobes were synthesized by T7 or SP6 polymerase with a DIG labeling mix (Roche, Tokyo) from a template containing the *p53* cDNA fragment. Micrographs were obtained with a BX50 optical microscope (Olympus, Tokyo). The micrographs were processed with Photoshop software (Adobe, San Jose, CA).

2.4. TUNEL (TdT-mediated dUTP-biotin nick-end labeling) staining

Medaka brains were fixed in 4% paraformaldehyde (prepared in phosphate buffer saline) overnight and embedded in paraffin. Each brain was sliced into 10-μm sections. Apoptotic cells were detected using a DeadEnd™ Fluorometric TUNEL System (Promega, Tokyo), according to the manufacturer's protocol.

3. Results

3.1. Distribution of proliferation zones and p53-expressing cells in brains of young medaka fish

To elucidate the molecular basis underlying cell proliferation in the medaka brain, we focused on medaka *p53* [6]. *p53* is expressed in proliferating and newly formed neurons of the adult murine brain [15]. To examine whether medaka *p53*-expressing cells were

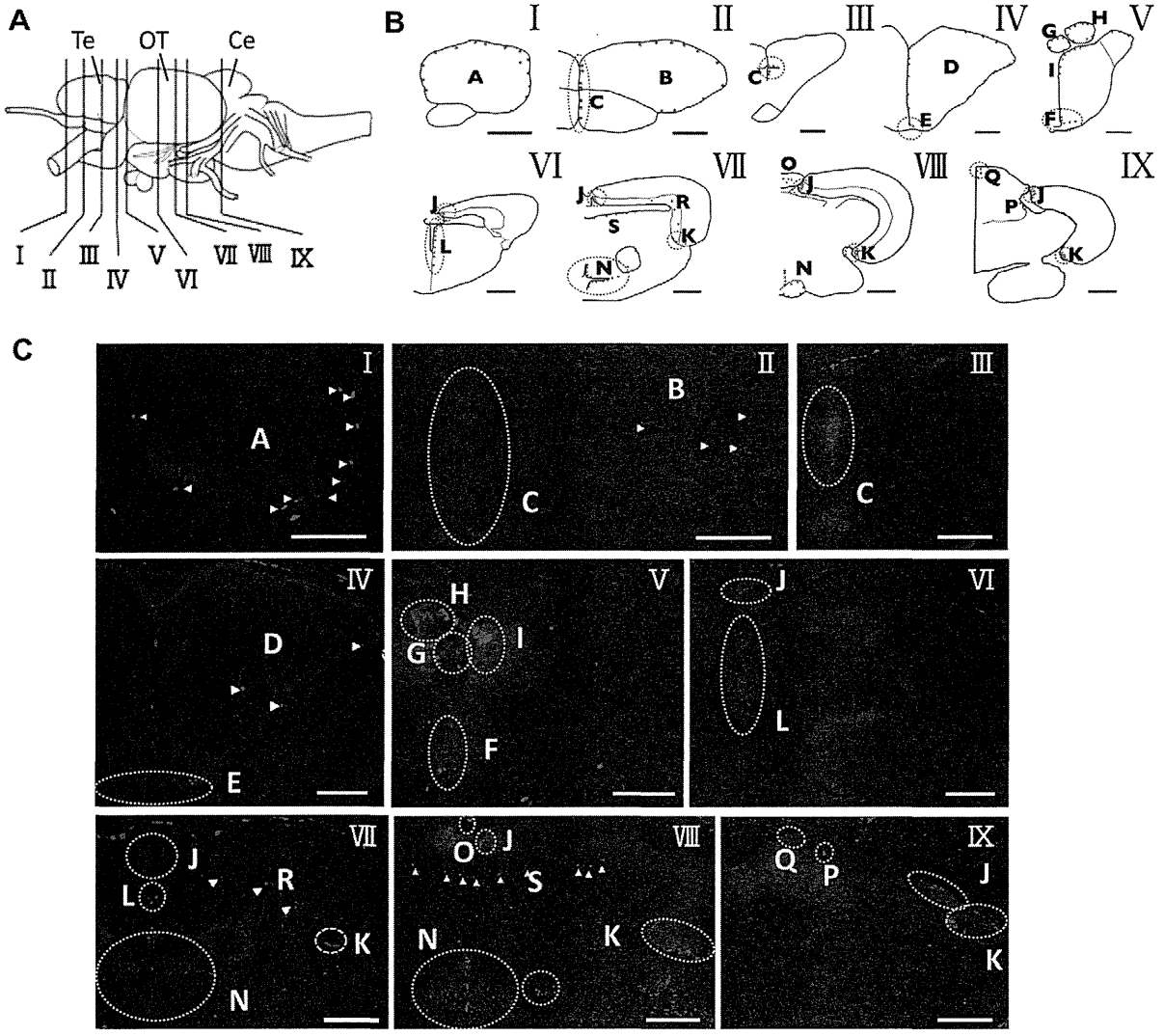

Fig. 1. Mapping proliferation zones in the brain of young medaka. (a) Schematic drawing of the lateral view of the medaka brain. The positions of sections I–IX are indicated by the lines. Te: telencephalon, OT: optic tectum, Cb: cerebellum. (b) Schematic representation of the distribution of the 18 proliferation zones. Red dots indicate proliferating cells. Zone A: marginal zones of the anterior part of the telencephalon, Zone B: marginal zones of the dorsolateral part of the telencephalon, Zone C: medial zones of the telencephalon, Zone D: dorsolateral part of the posterior part of the telencephalon, Zones E and F: preoptic area, Zone G: pineal body, Zone H: habenular nucleus, Zone I: ventromedial nucleus, Zones J and K: optic tectum, Zone L: anterior part of marginal zones of third ventricular zone, Zone N: hypothalamus, Zones O–Q: cerebellum, Zone R: periventricular grey zone (layer 3), and Zone S: Ependyme. Roman numerals in the panels correspond to section numbers shown in (a). Proliferation zones were determined according to the medaka fish brain atlas (Supplemental Fig. 1). (c) Distribution of BrdU-positive cells in the different proliferation zones. A magnified photo for zones P and Q (cerebellum) in panel XI is shown in Supplemental Fig. 2. Scale bars indicate 100 μm.

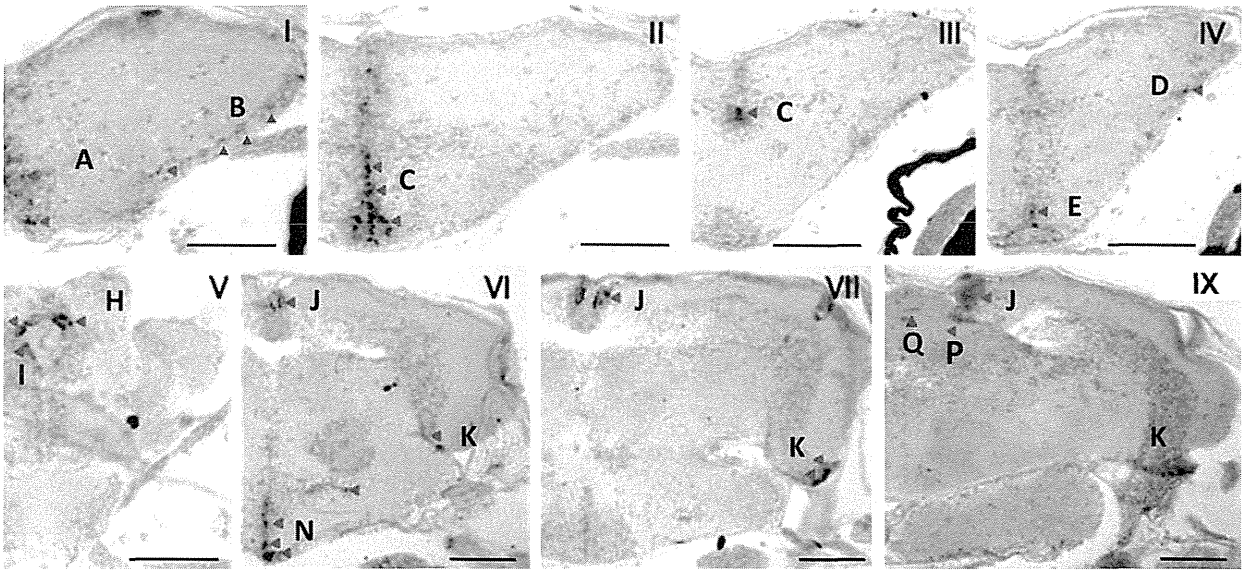

Fig. 2. Distribution of medaka *p53*-expressing cells in the brain of young medaka. Zones A–D: telencephalon, zone E: preoptic area, zone H: habenular nucleus, zone I: ventromedial nucleus, zones J and K: optic tectum, zone N: hypothalamus, zones P and Q: cerebellum. Scale bars indicate 100 μm.

present in the proliferation zones, we mapped the proliferation zones and performed *in situ* hybridization for detecting *p53* transcripts. In the present study, we used young medaka before they developed secondary sexual characteristics, because the smaller brain of the young medaka makes it easier to quantify newborn cells within a specific brain structure such as the telencephalon [2]. As a detailed description of the cell proliferation zones in the whole brain of sexually immature medaka is not available, we mapped the proliferation zones by identification of mitotic cells as determined by BrdU uptake. Based upon the distribution of DAPI staining and the medaka brain atlas [16], we identified the locations of the paraffin sections in the whole brain. We then mapped the BrdU-positive cells and identified 18 proliferation zones, A–L and N–S (Fig. 1, Supplemental Fig. 1). Sixteen zones (A–L and N–Q), were identical to those previously identified in sexually mature medaka [2]. In the present study, we could not confirm that there is a proliferation zone in the pituitary gland (zone M) previously identified in mature fish, as the pituitary gland is likely to be separate from the whole brain in the young fish. The 16 zones (A–L and N–Q) were mapped to the telencephalon (zones A–D), preoptic area (zones E and F), pineal body (zone G), habenular nucleus (zone H), ventromedial nucleus (zone I), optic tectum (zones J and K), marginal zone of the third ventricular zone (zone L), hypothalamus (zone N), and cerebellum (zones O–Q) (Supplemental Fig. 2). The two additional zones (R and S) were identified in the periventricular grey zone (layer 3) and ependyme, respectively, which were not previously found in the mature fish [2], suggesting that these two proliferation zones might disappear or integrate into the surrounding proliferation zones during the sexual maturation (Fig. 1). Next, to examine whether *p53* is expressed in proliferating zones in the medaka brains, we performed *in situ* hybridization. We demonstrated that medaka *p53* expressed selectively in at least 12 zones (zones A–E, H–K, N, P, and Q) (Fig. 2).

3.2. The p53 mutation had no effect on either the distribution of the proliferating zones or the number of proliferating cells

To examine whether *p53* is involved in cell proliferation in the medaka brain, we mapped proliferation zones using two *p53* mutant strains [6]. The $p53^{E241X}$ allele has a G to T substitution that changes Glu241 to a stop codon, and the $p53^{Y186X}$ allele has a T to A substitution that changes Tyr186 to a stop codon [6]. The two mutated *p53* genes encode truncated proteins that terminate within a DNA-binding domain. These proteins lack the nuclear localization signal and tetramerization domain required for full activity. Thus, these nonsense mutations probably lead to a null phenotype [6]. We found the 18 proliferation zones in the two mutant strains, $p53^{Y186X/Y186X}$ (Supplemental Fig. 3) and $p53^{E241X/Y186X}$ (data not shown), indicating that loss of *p53* has no effect on the distribution of proliferation zones. To examine whether the number of proliferating cells was affected by the *p53* null mutation, we counted the number of BrdU-positive cells in the entire telencephalon (zones A–D). There was no significant difference in BrdU-positive cells between the wild-type (WT) (average ± SE, 2316 ± 598; $n = 4$), $p53^{Y186X/Y186X}$ mutant (1849 ± 248; $n = 4$), or $p53^{E241X/E241X}$ mutant (1728 ± 366; $n = 3$) (Fig. 3D and F).

3.3. The p53 mutation led to decreased numbers of differentiated progenitors 1 week after BrdU exposure

To determine whether *p53* mutation affects survival and/or proliferation of progeny cells, we compared the distribution pattern of differentiated newborn cells in the brains of WT (Cab strain) and $p53^{Y186X/Y186X}$ mutant medaka. One week after BrdU exposure, BrdU-positive cells migrated from the proliferation zones (Fig. 3E) in the telencephalon of both WT and mutant strains, suggesting that there is no substantial difference in the migration pattern between the two strains. However, in some brain regions, such as the telencephalon (zone C) (Fig. 3E) and hypothalamus (zone N) (Supplemental Fig. 4B), the number of BrdU-positive neurons seemed to reduce in the mutant strain compared to the WT. Next, we quantified the number of BrdU-positive cells in WT (Cab strain), $p53^{E241X/E241X}$, and $p53^{Y186X/Y186X}$ in the telencephalon (zones A–D). In the WT, the number of BrdU-positive cells 1 week after BrdU exposure (6300 ± 535, average ± S.E, $n = 4$) increased over twofold (Fig. 3F), suggesting proliferation of the migrated progenitors. In contrast, there was no significant increase in BrdU-positive cells 1 week after BrdU exposure in $p53^{Y186X/Y186X}$ or $p53^{E241X/E241X}$ mutants (3596 ± 572 and 2378 ± 560, respectively). These results raised two possibilities: (1) the *p53* mutation enhanced cell death

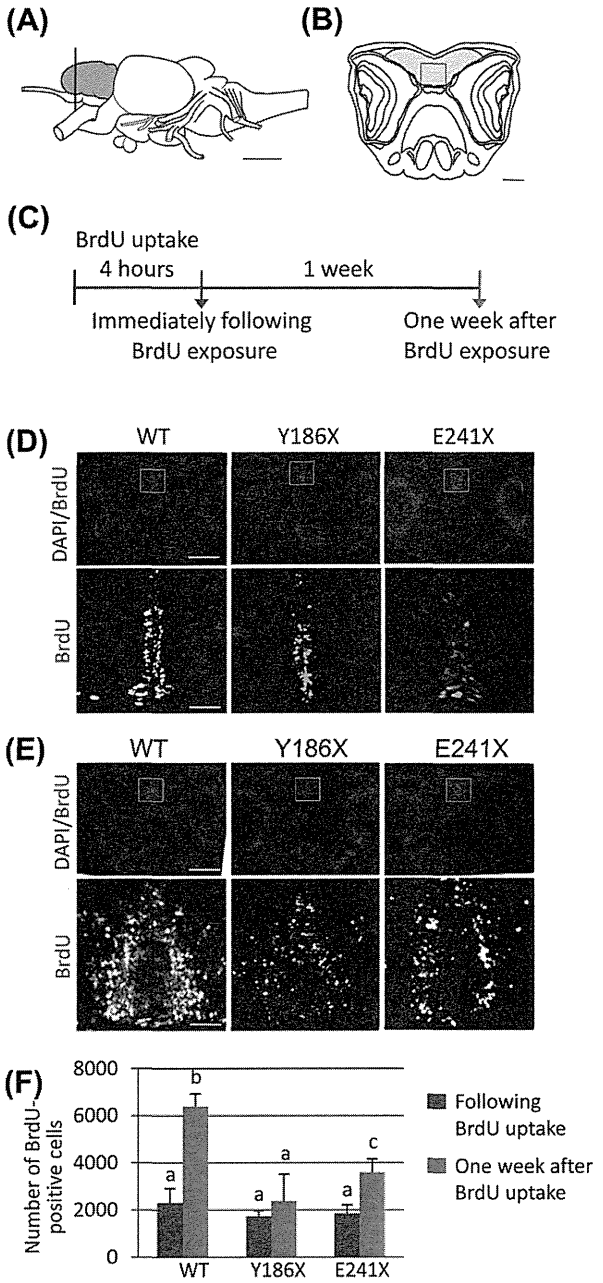

Fig. 3. Comparison of the distribution and number of newborn cells in WT and *p53* mutants. (A) Schematic drawing of the medaka brain and position of the telencephalon in the brain. (B) Schematic drawing of the transverse section of the medaka head. The section of images in (D) and (E), are indicated by a line and a square in (A) and (B), respectively. The pink area represents the medaka telencephalon. (C) The time schedule of this experiment. (D and E) Anti-BrdU immunohistochemistry of paraffin sections from wild-type (WT) medaka (Cab strain) and *p53* mutants (Magenta). Nuclei were stained with DAPI (Blue). The upper row indicates the transverse sections (Scale bars, 200 μm) and the lower row indicates the magnified view of the proliferation zone (Zone E; Scale bars, 40 μm), represented by the white rectangles in the upper row. (D) Immunohistochemistry was performed immediately after BrdU exposure. (E) Immunohistochemistry was performed 1 week after BrdU exposure. (F) Number of BrdU-positive cells in the telencephalon of WT and *p53* mutants medaka brains. Significant differences were observed between a and b, and b and c ($p < 0.01$ and $p < 0.05$, respectively; ANOVA with Bonferroni–Dunn post hoc test; $n = 3$–4 per group).

of differentiated progenitors (neuroblasts) or (2) the *p53* mutation repressed neuroblast proliferation and/or repressed differentiation of stem cells to an active, proliferating, neuroblast subpopulation.

To examine whether cell death was enhanced in the *p53* mutant strains, we compared TUNEL-positive cells in the telencephalon of WT and *p53* mutants. The number of TUNEL-positive cells was far less than the number of BrdU-positive cells in both WT and *p53* mutants, with no difference between the WT and *p53* mutants (Fig. 4A and B). We confirmed that TUNEL-positive signals were localized in nuclei stained with DAPI (Fig. 4A), and numerous TUNEL-positive cells were detected when using medaka pancreas sections, which are known to be susceptible to apoptosis [17] (Supplemental Fig. 4).

4. Discussion

In the present study, we demonstrated that the *p53* mutation did not affect the number of BrdU-positive cells immediately after BrdU exposure. In the SGZ of murine brains, adult neurogenesis originates from radial glia-like stem cells (Type 1 cells) through a proliferating stage (Type 2 cells) generating neuroblasts (Type 3 cells) and dentate granule interneurons [18]. Our finding strongly suggests that loss of medaka *p53* did not affect highly proliferating progenitors, which correspond to Type 1 and 2 cells. This seems inconsistent with a previous study indicating that genetic ablation of *p53* enhanced proliferation of stem cells in the adult murine brain [11]. There was no defect in stem cells in the *p53* mutant medaka brain. Most mice, zebrafish, and medaka with *p53* function defects develop without any obvious morphological defects [6,18,19–23], as p53 family proteins are redundant and can compensate for each other in various organs. Our results imply that other *p53* family members may compensate for a *p53* deficiency in medaka brain stem cells.

Furthermore, we showed that the number of newborn cells that migrate from the proliferation zones increased during the 1-week period after BrdU exposure in a *p53*-dependent manner. These data suggested that *p53* positively regulated the number of migrating progenitors, which may correspond to Type 3 cells (neuroblasts). Dividing neuroblasts are also found in the cerebellum (zone Q) of the zebrafish adult brain [22]. The shift in the distribution of BrdU-positive cells from the proliferation zone into the granule cell layers is accompanied by an increase in the number of labeled cells [23]. In the murine brain, there is some evidence for the proliferation of migrating neuroblasts [24], which originate from stem cells located in the SVZ of the lateral ventricles, moving along the rostral migratory stream. To determine which subpopulation of progenitor cells is regulated by *p53*, it will be crucial to characterize the subtype and maturation sequence of progenitor cells in the medaka brain.

Positive regulation of *p53* in adult medaka brain neurogenesis appears to be the opposite of what is observed in murine *p53* mutants [11,20], where *p53* negatively regulates neurogenesis. One possible explanation is that the p53 N-truncated isoform, which has the opposite effect, may function in the medaka brain. In mice and zebrafish, the *p53* family genes (including *p63* and *p73*) have 2 isoforms—full length and N-truncated—with an alternative transcriptional start site [10,20,25]. Because the latter isoform lacks a transactivation domain, it is thought to function in a dominant-negative fashion to inhibit the transcriptional activity of full-length p53 family members. In the murine brain, p53 family proteins interact with each other in a cell-type/stage-specific manner and coordinated expression of the two isoforms is required for stem cell maintenance in adult neurogenesis [10,20,25,26]. As positive regulation of *p53* in neurogenesis has not been indicated in the murine brain, a *p53* study using medaka may shed a light on a novel mechanism underlying adult neurogenesis.

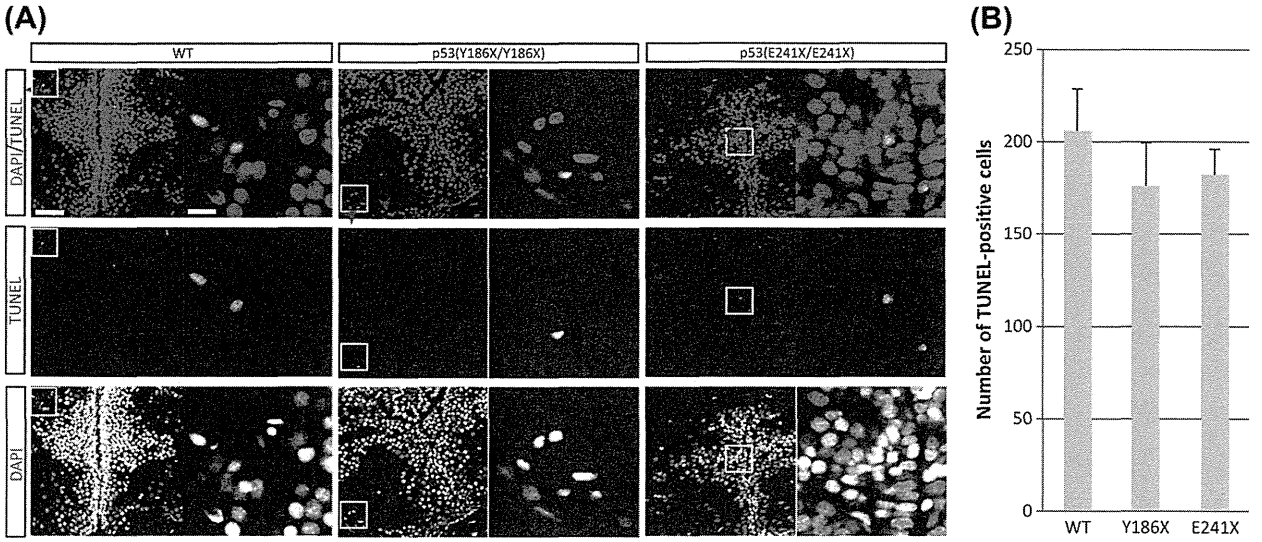

Fig. 4. Cell death in the telencephalon of young medaka. (A) Confocal images show double-labeling of TUNEL (Magenta) and DAPI (Green) in Zone D. Red arrow head indicate TUNEL-positive cells. For each strain, images in the right column are the magnified images of the region outlined by the white rectangle in the left column images. Scale bars indicate 80 μm (Left) and 20 μm (Right) (B) quantification of TUNEL-positive cells. No significant difference was detected (ANOVA with a Bonferroni–Dunn post hoc test; $n = 3–4$ per group).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.125.

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