Retinitis pigmentosa (RP) is a progressive degenerative disorder characterized by night blindness, progressive loss of peripheral vision and finally a vision acuity handicap that may progress to complete blindness. X chromosome-linked RP (XLRP), which always presents with severe symptoms, early onset and rapid deterioration [1], accounts for 6-17% of familial RP cases [2-5]. Five genetic loci for XLRP, including RP2 (MIM 312600) [6], RPGR (MIM 312610) [7], RP6 (MIM 312612) [8], RP23 (MIM 300424) [9], and RP24 (MIM 300155) [10], have been mapped on the human X chromosome. According to the linkage analysis, RPGR is considered to account for 56-90% of XLRP while RP2 accounts for 10-20% [11-14]. These two major loci have been successfully cloned [15-17]. ORF15 is a new exon at the C-terminus of RPGR, which has been identified in the original intron 15 in European patients [18] and has been demonstrated to be a mutation hot spot in different populations [19-21].

An RP family that has a typical X-linked mode of inheritance is defined as a confirmed XLRP pedigree. The “multiplex RP” family refers to the RP family with more than one affected individual, but where there is insufficient information to indicate any specific Mendelian pattern of inheritance. A simplex case is defined when an individual RP patient has no family history and consanguinity in the pedigree. Genetic subtyping is an important basis for counseling and molecular screening. However, a large proportion of RP patients are unable to recall their affected relatives, and thus it is hard to establish the segregation pattern. Data on genetic subtypes of RP from different groups/countries have varied considerably, partly due to the application of different classification criteria [22]. In addition to genetic subtyping, clinical subtyping has been undertaken by determining the clinical characteristics in different forms of RP, and it has also been demonstrated that mild or severe myopia, early onset, and severe RP phenotype represent the clinical risk factors for XLRP [23]. Many previous studies have shown that high myopia is frequently observed in carriers and male XLRP patients [24,25]. These findings support the notion that routine ophthalmic examinations may be useful for genetic classification of affected patients, and for the identification of other family members who are at a high risk of having the early stage of RP. The aim of the present study was to identify mutations in RPGR and RP2 genes in a series of myopic RP families including 3 confirmed XLRP, 29 multiplex RP, and 5 simplex RP, and to determine the association between the phenotypic changes and the mutations.
was no male-to-male transmission and consanguinity in at least three generations, there was at least one affected son of an unaffected mother, there were severe clinical symptoms in affected males, and there were wide-spectrum of symptoms in obligate carriers but without similar manifestation in all affected individuals. An individual was considered to be affected when (1) the abnormalities were clinically evident according to an examination and diagnosis by an ophthalmologist, or (2) if there was a clear report of a highly suggestive manifestation such as night blindness and progressive visual loss. The multiplex RP was defined as above. Among the 37 families, three families (family C, E and F) had typical X-linked mode of inheritance and thus were defined as having confirmed XLRP (Figure 1). Among the other 34 families, 29 were multiplex RP without remarkable family history to establish the segregation pattern but without evidence of male-to-male transmission, and the other five were isolated RP cases. In addition, these 34 families had at least one patient with myopia greater than -3.0D (equivalent spherical diopters) in both eyes. 118 healthy volunteers (age range 20-74 years, 68 males and 50 females) were included as controls in the study.

A written informed consent was obtained from each subject or the responsible adult of the family, and the study was approved by the local ethics committee. The protocol of the study adhered to the tenets of the Declaration of Helsinki. Ophthalmic examinations, including a routine ophthalmic examination,Goldmann perimetry, full-field electroretinography (ERG) and color fundus photography, were performed. Available information on family history and pedigrees were obtained through personal interviews with patients or their family members.

Comprehensive screening in RPGR and RP2: Blood of the patients from 37 unrelated families were collected (Figure 1). DNA was extracted following provided protocols (Wako,

Figure 1. Pedigrees of the families A, B, C, D, E, and F. Closed symbols indicate individuals with RP and open symbols indicate unaffected subjects. Dotted circles indicate female carriers. The bars over the symbols indicate personally examined subjects. Arrows indicate probands. Slash indicates deceased person.
Japan). The coding fragments and intron/exon boundaries of RPGR gene, including exon1-19, exon15a, exon ORF15, and RP2 gene were amplified by polymerase chain reactions (PCR) and sequenced according to the protocols as described previously [15,17,21,26]. To verify the products before sequencing, PCR products were electrophoresed on agarose gels, and then purified by a PCR Centrifugal Filter Device (Amicon, Bedford, MA). Purified DNA samples were used for cycle sequencing by the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). To examine exon ORF15, we used ABI PRISM dGTP Big Dye Terminator Cycle Sequencing FS Reaction kit (Applied Biosystems, Foster City, CA) with the published primers [15,17,21,26]. Reactions were carried out on ABI 310 Genetic Analyzer or 3100 Automated Sequencer (Applied Biosystems). Identified mutations were confirmed by the same procedure, and frameshift mutations were analyzed for predicted protein changes by DNASIS software (HITACHI, Japan). In addition, direct sequencing was performed on exon ORF15 in 118 volunteers, and on RPGR (exon 1-19 and exon15a) and RP2 in 80 volunteers.

RESULTS

Mutations in RPGR and RP2 genes and sequence variations: Overall, three mutations in ORF15 of RPGR gene and one mutation in RP2 gene were identified (Figure 2). Pathogenic mutations were found in two of the three confirmed XLRP families and three of the 29 multiplex RP families. No mutation was identified in 5 simplex RP patients. Two unrelated families (family A and B) shared a same mutation, g.ORF15+652-653delAG, which was predicted to create a premature stop codon at 248 (Glu217fsTer248). Two other frameshift mutations were detected in family C.

<table>
<thead>
<tr>
<th>Family</th>
<th>Mutation</th>
<th>M/F</th>
<th>Age(y)</th>
<th>Onset(y)</th>
<th>BCVA: RE/LE</th>
<th>Refractive error (D)</th>
<th>ERG</th>
<th>Visual Field</th>
<th>Fundus</th>
</tr>
</thead>
<tbody>
<tr>
<td>A II:3</td>
<td>F</td>
<td>66*</td>
<td>-</td>
<td>1.0/0.6</td>
<td>+0.25D=cyl-1.5Dax50</td>
<td>reduced</td>
<td>full</td>
<td>TLR</td>
<td></td>
</tr>
<tr>
<td>A II:3</td>
<td>M</td>
<td>42</td>
<td>11</td>
<td>0.3/0.3</td>
<td>-0.25D=cyl-0.25Dax20</td>
<td>extinguished</td>
<td>V-4, 15-20°</td>
<td>PBSL, MD</td>
<td></td>
</tr>
<tr>
<td>A II:4</td>
<td>M</td>
<td>41</td>
<td>8</td>
<td>0.8/0.8</td>
<td>-5.0D=cyl-0.25Dax170</td>
<td>extinguished</td>
<td>V-4, 15°, AS</td>
<td>MF, PP</td>
<td></td>
</tr>
<tr>
<td>B II:6</td>
<td>F</td>
<td>58</td>
<td>35</td>
<td>0.7/0.01</td>
<td>-4.25D</td>
<td>extinguished</td>
<td>V-4, 10°, RS</td>
<td>PBSL</td>
<td></td>
</tr>
<tr>
<td>B II:6</td>
<td>F</td>
<td>30</td>
<td>-</td>
<td>1.0/1.0</td>
<td>-4.0D=cyl-0.5Dax180</td>
<td>normal</td>
<td>NA</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>C II:2</td>
<td>F</td>
<td>47</td>
<td>-</td>
<td>7/0.7</td>
<td>-14.0D=cyl-1.00D Ax110</td>
<td>reduced</td>
<td>n.d</td>
<td>MF</td>
<td></td>
</tr>
<tr>
<td>C II:4</td>
<td>M</td>
<td>43</td>
<td>20</td>
<td>CF/0.05</td>
<td>-3.75D=cyl-1.00Dax50</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>C IV:1</td>
<td>F</td>
<td>19</td>
<td>-</td>
<td>1.0/1.0</td>
<td>-9.0D=cyl-0.5Dax170</td>
<td>normal</td>
<td>n.d</td>
<td>MF, PP</td>
<td></td>
</tr>
<tr>
<td>D II:3</td>
<td>M</td>
<td>33</td>
<td>10</td>
<td>0.4/0.4</td>
<td>-0.75D</td>
<td>extinguished</td>
<td>V-4, 10-15°, RS</td>
<td>PBSL</td>
<td></td>
</tr>
<tr>
<td>D IV:4</td>
<td>F</td>
<td>9</td>
<td>-</td>
<td>1.0/1.0</td>
<td>-9.25D=cyl-2.00Dax180</td>
<td>reduced</td>
<td>V-4, 45-55°</td>
<td>TLR</td>
<td></td>
</tr>
<tr>
<td>E II:5</td>
<td>F</td>
<td>52</td>
<td>-</td>
<td>1.0/1.0</td>
<td>-6.25D=cyl-4.00Dax180</td>
<td>reduced</td>
<td>NA</td>
<td>PP</td>
<td></td>
</tr>
<tr>
<td>E III:5</td>
<td>F</td>
<td>28</td>
<td>-</td>
<td>1.0/1.0</td>
<td>-4.25D=cyl-2.50Dax180</td>
<td>normal</td>
<td>full</td>
<td>PBSL</td>
<td></td>
</tr>
<tr>
<td>E III:6</td>
<td>M</td>
<td>25</td>
<td>7</td>
<td>0.6/0.6</td>
<td>-2.75D=cyl-0.75Dax175</td>
<td>extinguished</td>
<td>V-4, 20-30°</td>
<td>PBSL</td>
<td></td>
</tr>
</tbody>
</table>

BCVA represents best corrected visual acuity, RE represents right eye, LE represents left eye, CF represents count fingers, RS represents ring scotoma, AS represents arcuate scotoma, MD represents macular degeneration, MF represents myopic fundus, PBSL represents pigmentbone spicule-like, PP represents peripheral pigmentation, TLR represents tapetal-like reflex, N represents normal, NA represents not available, n.d represents not determined. Asterisk represents the II:3 in family A were examined at 33 year old.
These two mutations were predicted to result in the early termination of the proteins (Glu161fsTer183 and Glu224fsTer248, respectively). Family E had a nonsense mutation (358C>T, Arg120Ter) in RP2 gene. All mutations co-segregated together with affected patients and carriers in each family. None of these mutations was found in normal controls or in the normal relatives in XLRP families. Nucleotide positions were located based on GenBank sequence (ORF15, accession number AF286472; RP2, accession number NM_006915). The frameshift mutations were designated according to the following example: ORF15Glu161fsTer183 refers to a frameshift mutation, in which Glu161 is the first amino acid altered, and the termination of the open reading frame locates at residue 183.

Phenotypes of families with mutation: The clinical data of the subjects in the families with mutations in RPGR and RP2 genes were summarized in Table 1. Two unrelated families (A and B) shared the same deletion of 652-653 in ORF15 (g.ORF15+652-653delAG).

Two affected brothers (III:3 and III:4) and their mother, the carrier, (II:3) in family A (Figure 1) were reported previously [27]. The 42-year-old affected male (III:3) noticed reduced visual acuity at the age of 9. At the age of 18, fundus examination showed typical RP and ERG showed an extinguished response. Reduced visual field and abnormal dark adaptation were also observed [27]. His best corrected visual acuity was 0.3/0.3 with mild myopia. Fundus examination showed bone spicular pigmentations and macular degeneration (Figure 3A). The visual fields were constricted. ERG showed an extinguished response, with best corrected visual acuity of 1.0/0.8, but no visual fields were apparently defected. However, the visual field examination revealed scotoma when she was 54 years old, and ERG demonstrated a reduced response, with best corrected visual acuity of 1.0 and 0.3. When she was 58 years old, her best corrected visual acuity was reduced to 0.7 with a refraction of +0.25D and 0.01 (uncorrectable). Cataractous changes affected the left eye more markedly than the right. The visual field examination also showed ring scotoma, and ERG revealed an extinguished response (Figure 3C). Her daughter (III:6), a 30-year-old heterozygote carrier, was asymptomatic with normal fundoscopic examination and ERG.

Phenotypic changes in three families: In family C (Figure 1), a female carrier and two affected males (III:2, III:4, and IV:1) were assessed. They all had high myopia with vari-
able astigmatism. The carrier III:2 did not report night blindness. ERG showed a subnormal response (Figure 3D). The fundus examination revealed myopic chorioretinal degenerations (Figure 3D), and a few bone spicules were seen in the peripheral fundi. Although the individual III:4, aged 43 years, was not examined in the study, his medical record showed that he had an onset of symptoms since he was 20 years old, and had bilateral reduced central vision (count fingers, CF/0.05) in recent health examinations. The individual IV:1 was diagnosed as having RP in a medical examination when she was 10 years old, but she had no complaint of vision impairment and night blindness. The fundus examination showed a few spicule-like changes in the peripheral fundi. ERG showed a normal response.

In family D, an affected male (III:3) and his daughter, the carrier (IV:4), were assessed. The proband had an early onset of night blindness (10 years of age). ERG showed an extinguished response (Figure 3E), and Goldmann perimetry showed concentric constrictive visual fields. Bone spicule-like changes were found in the midperipheral retina during the fundus examination (Figure 3E). His daughter (IV:4) was found to have myopia and astigmatism by skiascope when she was 3 years old. ERG showed a negative-type response (Figure 3F). Perimetry examinations did not show any abnormalities, whereas the fundus examination demonstrated a tapetal-like reflex in bilateral fundi (Figure 3F).

In family E, an affected male (III:6) and two female carriers (II:5 and III:5) were assessed. The affected male reported night blindness at his childhood while two carriers had no complaint of night blindness. Individual III:6 was demonstrated to have an extinguished ERG response (Figure 3G) and constrictive visual fields. His mother, II:5, had a subnormal ERG response while another carrier (III:5) demonstrated a normal ERG response. Fundoscopy showed peripheral pigmentation.

Figure 3. Representative fundus photographs and ERG results of the XLRP individuals. A: Family A, patient III:3: Fundus photograph showing bone spicule-like pigmentation (arrowhead) and macular degeneration. B: Family A, patient III:4: Fundus photograph showing leopard fundus and a few pigmentary changes (arrowhead) in the midperipheral retina. C: Family B, patient II:6: Fundus photograph showing extensive bone spicular changes and the maximal combined response (right). D: Family C, patient III:2: Fundus photograph from a carrier showing extensive myopic chorioretinal degeneration with peripapillary atrophy (left) and the maximal combined response (right). E: Family D, patient III:3: Fundus photograph showing the accumulation of bone spicules in the midperipheral fundus (left) and the maximal combined response (right). F: Family D, patient IV:4: Fundus photography of a carrier showing tapetal-like reflex (left) and maximal combined response (right). G: Family E, patient III:6: Fundus photography showing bone spicular pigmentations in midperipheral fundus (left) and maximal combined response (right).
in all the three individuals but it was more apparent in the affected male (Figure 3H).

**DISCUSSION**

RP is the most frequent genetic disorder in the human eye. To date, at least 30 RP-related genes or loci have been mapped. However, almost 50% RP patients were multiplex and simplex cases in different populations [5,22,28,29]. Since families are usually small, and expressivities of the disease vary in individuals, the patients may present a wide spectrum of phenotypes from complete normal to severe symptoms. Several genetic alterations are responsible for the existence of a large number of multiplex and simplex RP, including autosomal recessive inheritance, skewed X chromosome inactivation (X-inactivation) of carriers in XLRP families, germinal mosaicism and/or somatic mosaicism and partial penetrance of dominant RP.

Many previous studies have reported that most XLRP patients are myopic and myopia is much more prevalent among XLRP than other forms of RP, suggesting that a correlation may exist between XLRP and myopia [24,25]. In a previous study, however, myopia, early onset, and relative severe manifestations were considered to be the risk factors for XLRP based on the evaluation of an RP outpatient [23]. We have also observed that high myopia is frequently seen in the XLRP patients as well as carriers with proven mutations [21,30]. This finding prompted us to perform a mutational screening which included XLRP families and the myopic RP families whose family histories were not sufficiently remarkable to be classified as a defined inheritance mode or isolated. It is not surprising that two of the three confirmed XLRP families were found to have mutations. Moreover, in the 29 multiplex XLRP families, three had ORF15 mutations. Since mutations in RPGR and RP2 genes account for almost all the XLRP, we hypothesize that a considerable proportion of myopic RP patients without a remarkable family history are actually having X chromosome inheritance. It must be noted that not all RP individuals who had a proven mutation as shown in the present study had severe myopia and the family members in the same families with a mutation had remarkably different severity of myopia. However, high myopia was frequently seen in those individuals, especially in the obligate carriers. Further prospective cohort studies are required to elucidate if high myopia is correlated with XLRP.

Direct PCR-sequencing is a more sensitive and discriminating method of identifying genetic defects than the cleavage method, such as PCR-single strand conformational polymorphism. PCR-sequencing of ORF15 is technically challenging, although several investigators have extended the method and have developed new strategies to amplify the exon ORF15 [31,32]. In the present study, we used two protocols to amplify the exon ORF15 as described previously [21,26], and it was revealed that three of the four mutations in Japanese XLRP patients were in exon ORF15. Moreover, all the three ORF15 mutations were reported for the first time in Japanese XLRP patients, although they have been previously reported in other populations [18,26,33-35], suggesting that they are common mutations in XLRP patients. Recently, as more mutations in RPGR and RP2 genes are reported in XLRP patients [36-39], and important advances on the mystery of RPGR function are disclosed [40,41], the accumulative data on distinct RPGR mutations in XLRP patients from different populations may provide important clues for the potential function test or gene therapy, which will become practicable in humans in the future.

Another important finding from the present study was the absence of RPGR and RP2 mutations in family F with X chromosome inheritance. In addition to RPGR and RP2, three other loci on the X chromosome, i.e. RP6 on Xp21.3-p21.2, RP23 on Xp22 and RP24 spanning on Xq26-q27 that may be responsible for XLRP have been reported, but they are poorly clarified. Most recently, a new genetic locus for an XLRP on chromosome Xq28 has been identified [42]. We are currently planning to expand the family data to perform a linkage analysis and additional clinical examinations of this pedigree in order to identify the underlying gene(s) associated with XLRP.

The detailed phenotypes of the patients with mutations in the present study were consistent with XLRP. Although the ORF15 mutations have been reported previously [18,26,33-35,39], to our knowledge, only Andreasson et al described in detail the phenotypes of two Swedish XLRP families with the ORF15 mutations which were also identified in families A, B, and D (g.ORF15+673_674delAG and g.ORF15+673_674delAG) in the present study. Similar to their findings, all the three patients (A:III:3, A:III:4, and D:III:3) in the present study demonstrated an extinguished ERG response and a constricted visual field. However, it appears that patients in the present study showed a better remnant visual acuity than the age-matched patients in their report. They also reported a typical RP fundus change and a remarkably reduced retinal function in all the three female carriers. However, among four of our female carriers (A:II:3, B:II:6, B:III:6, and D:IV:4) in the present study, one (B:III:6) showed completely normal phenotypes, two (A:II:3 and D:IV:4) demonstrated tapetal-like reflex in the fundus, and only B:II:6 was found to have pigment bone spicule-like change in the both eyes. Additionally, the four female carriers showed a wide spectrum of clinical features, from normal to severe symptoms. For example, B:II:6 demonstrated a markedly reduced visual acuity (0.7/0.01), an extinguished ERG response and constricted visual field, while other three showed mild phenotypes or even a completely normal visual acuity and ERG response. The broad-spectrum phenotypes in female carriers may be explained by the favorite early X-inactivation during embryogenesis [43]. Tapetal-like reflex was found in two unrelated carriers (A:II:3 and D:IV:4), indicating that the tapetal-like reflex is a clinical sign of XLRP carrier status. Rozet et al reported 14 XLRP families with constant and severe clinical phenotypes in female carriers, and hypothesized that the form of XLRP might be partial dominant which was distinct from the general viewpoint [34]. Most recently, Sullivan et al identified RPGR mutations in two RP families which were misconstrued as autosomal dominant RP because of the affected female carriers [44]. A noteworthy is the fact that the female...
carriers in these two studies had delayed onset and milder severity compared with male patients. It has been well established that X-inactivation occurs, coupled with cellular differentiation, during the early stages of female embryogenesis, and females have varying X-inactivation ratios. Only a very small number of females are found to have skewed X-inactivation. A previous study by Rozet et al showed no preferential X inactivation in several female carriers with a non-recordable ERG response [45]. However, this finding did not reflect the general status of X-inactivation because the methods based on methylation analysis is an indirect assessment of X-inactivation status and the analysis was performed on only peripheral blood samples, which could not represent the X-inactivation patterns in retina. The random nature of X-inactivation and the variable clinical features of female carriers with XLRP remain to be elucidated. Regardless of the true status of X-inactivation, the clinical features of female carriers in these previous studies seemed more severe than those of the female carriers in the present study [34,39].

Two age-matched brothers in family A showed apparent differences in visual impairment, suggesting an intra-familial variance. Unidentified factors, such as environmental factors, individual living habits, and additional modifier genes might impact on the clinical presentation of this disease.

In conclusion, we identified four mutations in \( \text{RPGR} \) and \( \text{RP2} \) genes in five unrelated Japanese RP families and documented the phenotypic changes which were distinct from a previous study. \( \text{RPGR} \) and \( \text{RP2} \) mutations may be responsible for a substantial proportion of multiplex RP patients with myopia. In other words, myopia may be an indicator for XLRP in multiplex RP family. Therefore, it is suggested that mutational analysis of \( \text{RPGR} \) and \( \text{RP2} \) may help to identify the causative mutation in a proportion of multiplex RP patients with myopia.

**ACKNOWLEDGEMENTS**

We thank the XLRP patients and their families for their participation in this study. We would like to express our gratitude to Hai-Ying Shen, MD & PhD, and Feng Gu, PhD, for their helpful suggestions and Dr. Harry HX Xia for language assistance. This study was supported partly by JSPS Grant-in-Aid for Scientific Research (C) 17591843.

**REFERENCES**


