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Analysis of Mutations of Retinitis pigmentosa by Sequencing

Nuray ALTINTAŞ[1]* Özge SARICA YILMAZ[1] Ali SOYLU[1] İlker BİÇER[2]
[1]Celal Bayar University, Faculty of Medicine, Department of Medical Biology, Manisa, Turkey
[2]Manisa State Hospital, Ophthalmology Clinic, Manisa, Turkey

*Corresponding Author:
E-mail:naltintas35@gmail.com

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Abstract

In our study, we aimed to provide the first source of regional data obtained from molecular investigations by sequencing of selected exons of RPE65 and RPGR genes in Retinitis pigmentosa (RP) patients in Manisa province. Ethical committee approval was obtained and the study group consisted of 100 healthy volunteers with no clinical history of RP and RP with approved clinical manifestations in Ophthalmology Clinic of Manisa State Hospital. DNA samples were analyzed by Sequential Analysis and SeqScape software using ABI Prism 310 Genetic Analyzer and mutation tables. As a consequence of sequencing the sequence in the RPE65 gene in four patients with RP, two mutations known to be associated with the RPE65 gene have emerged in the BBS.E352E G> A Glu352Glu (Syn; 1056G> A) and R91Q G> A homozygote mutations were detected.Two RP patients had Bardet-Biedl syndrome.As a result of the sequence screening gene in RPGR , ORF15+1478 T>A heterozygote mutation in ORF15 exon of RPGR gene in 2 women patients , ORF15+1643 C>T and ORF15+1677 G>A polymorphisms in ORF15 exon of RPGR gene in 2 men patients were detected. Our results are originally given as regional first in Manisa and its region.

Keywords: RP, DNA Sequencing, ORF15, RPGR,RPE65

INTRODUCTION

Retinitis pigmentosa (RP) is a clinically and genetically heterogeneous group of retinal dystrophies characterized by photoreceptor cell degeneration.The prevalence of RP is about 1/4000 ~1/5000 according to studies in different populations[3]. RP is an inherited retinal degeneration that affects approximately one in 3,500 individuals,with an estimated total of 1.5 million patients worldwide. RP is caused by progressive loss of rod and cone photoreceptors. Patients experience night blindness followed by loss of visual fields, usually culminating in legal and often complete blindness. Clinical hallmarks of RP include bone spicule deposits, attenuated retinal blood vessels, optic disc pallor, visual field loss, and abnormal, diminished or nonrecordable electroretinographic responses (ERG) [1].

Patients with RP usually present with a loss of night vision and complain of progressive constriction of the visual field. Eventually, central vision is affected and worsens. Clinical features such as age of onset and rate of progression vary greatly between individuals. The identification of causative genetic variants is expectedto be the starting point of RP treatment. Genetic counselling and gene therapy or other patient-specifictreatment options can be suggested based on geneticbackground. In addition to treating the disease, molecular diagnosis is helpful for informing the differential diagnosis. Hereditary retinal dystrophies have a slowly progressive nature and complete clinical features may not be present at the time of examination. Therefore, many other hereditary retinal dystrophies may be confused with RP at certain phases of disease progression. However, the genetic heterogeneity of RP is a hurdle for the easy application of molecular diagnosis. At the time of September 2011, 53 genes and 7 mapped loci are known to be associated with nonsyndromic RP (RetNet). These genes show considerable phenotypic variability and multiple inherited retinal disease shares certain causative genes with RP [5].

Over 50 genes have been identified to cause RP, but still only explain no more than half of the clinical cases . Therefore, there has been limited success with approaches of screening of known candidate genes for RP by conventional Sanger sequencing. Fortunately, exome sequencing technique has come to the aid by enabling the identification of disease-associated mutations by sequencing the whole exome of a small number of affected individuals [4].

RP can be inherited in an autosomal dominant (adRP),autosomal recessive (arRP), or X-linked (XLRP) manner, with rare digenic and mitochondrial forms. RP also is associated with several syndromic disorders, such as Bardet-Biedl and Usher syndrome.To date, mutations in 23 genes are known to cause adRP, mutations in 36 genes cause arRP, and mutations in 3 genes cause XLRP. These genes encode proteins involved in various retinal functions, including phototransduction, photoreceptor outer-segment structure, and pre-mRNA splicing. Despite the large number of RP genes, mutations cannot be identified in 30 to 35% of patients with adRP. X-linked forms of RP account for 10 to 20% of all RP families. Mutations in the RPGR gene are the most common causes of XLRP, accounting for over 70% of XLRP. RPGR mutations also may account for more than 15% of simplex (isolated) male subjects with RP. RPGR localizes to the connecting cilium of photoreceptors and is believed to have a role in protein transport. Approximately 60% of diseasecausing mutations in RPGR are found in ORF15. ORF15's sequence is highly repetitive and purine-rich. This type of sequence often promotes polymerase arrest and slipped strand mispairing, thus making ORF15 a hotspot for mutations. ORF15 is a component of alternate splice variants of RPGR that are expressed predominantly in the retina, as is exon 9a [1].

RPE65 gene was discovered through its protein product that forms a complex with an antibody raised against human retinal pigment epithelium . The cDNA sequence predicts a protein with 533 amino acid esidues and a molecular mass of '61 kDa . The protein is associated with the endoplasmic reticulum of the retinal pigment epithelium in vertebrates. The protein is associated with the endoplasmic reticulum of the retinal pigment epithelium in vertebrates . Although the biochemical function of the protein product is currently

obscure, the tissue-specific expression of the RPE65 gene made it an attractive candidate as a cause for some retinal degenerations because the retinal pigment epithelium has an essential role in maintaining the viability of the neighboring photoreceptor cells. In particular, enzymes in the endoplasmic reticulum are responsible for the recycling of the chromophore used by photoreceptor opsins [2).

MATERIALS and METHODS

In the present study, disease-associated mutations were identified in a large Turkish family with RP complicated with subcapsular cataract , corneal thinning and high myopia , Mistagmus (Figure 1) using the exome sequencing techniques.

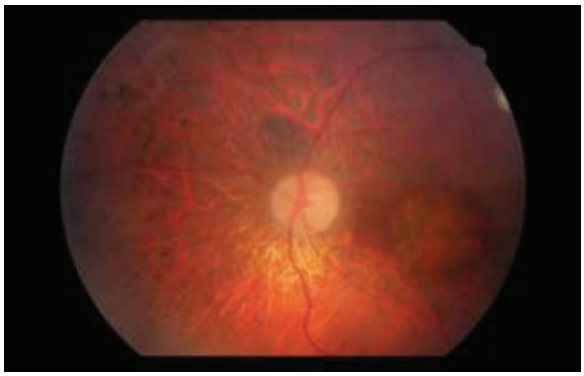

Figure 1. Fundus photograph of the RP patient. Fundus photograph of showing typical changes of RP including waxy yellow appearance of the optic disc, attenuation of retinal arteries and bone-spicule pigment deposits in the mid periphery of the retina.

DNA Extraction

The total genomic DNA (gDNA) was extracted using RTA-DNA Isolation Kit (Gebze/Kocaeli, Turkey) according to manufacturer instructions from peripheral blood. DNA samples were stored at -20 C until used. DNA integrity was evaluated by 1% agarose gel electrophoresis.

PCR products of exons 15;1,3,13 in ORF15 of RPGR gene, exons4 ,5,10,111,13 of RPE65 gene were amplified by polymerase chain reaction (PCR) (Figure2-A, Figure 3-B).

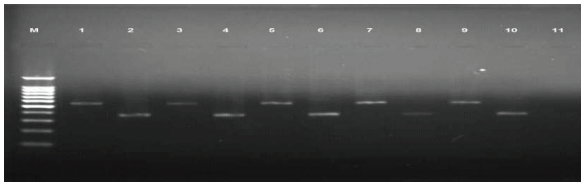

Figure 2. A.PCR products of exon 15;1,3,13 in ORF15 of RPGR gene

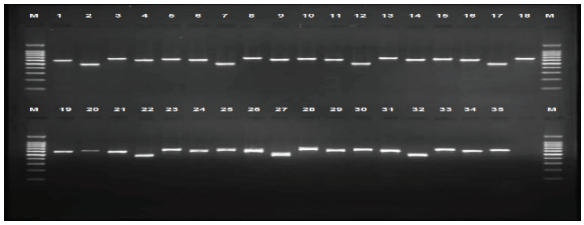

Figure 3. B.PCR products of exon 4,5,10,11,13 of RPE65 gene.

PCR reaction for RPE 65 gene, Forward and Reverse primers and Tm ratios for exons, PCR product bp are as follows:

RPE65-Exon 4-Forward(22bp)(Tm:65,08 oC)
CCTAGCACTGTGTCCCACCTGC PZR: 543 bp
RPE 65- Exon 4-Reverse (19 bp) (Tm: 63,22 oC)
CTGCCCTGCTTGGTCACCC PZR: 543 bp
RPE 65-Exon 5-Forward (21 bp) (Tm: 61,16 oC)
TGCAGTCCATTTGGAGCTTGG PZR: 435 bp
RPE 65-Exon 5-Reverse (20 bp) (Tm: 62,98 oC)
TGGCACCTGTGCTTTCCCAG PZR: 435 bp
RPE 65-Exon 10-Forward (23 bp) (Tm: 62,06 oC)
TGGATCTGCACTATTCACCGAGG PZR: 600 bp
RPE 65-Exon 10-Reverse (20 bp) (Tm: 62,48 oC)
CTGTGTGGGCGGGAGGAAAT PZR: 600 bp
RPE 65- Exon 11- Forward (27 bp) (Tm: 62,94 oC)
TTCAGCTTACAGAGCTGTTTGTGAGAA PZR: 548 bp
RPE 65- Exon 11- Reverse (21 bp) (Tm: 63,56 oC)
TCCTGCAGTTCCTCCCTGCAT PZR: 548 bp
RPE 65- Exon 13- Forward (22 bp) (Tm: 61,45 oC)
TGCTCCATCGTGACACCAAATG PZR: 576 bp
RPE 65- Exon 13- Reverse (27 bp) (Tm: 63,01 oC)
TCCTAAGCATGTGCTCTATTTCGTAGCPZR: 576 bp

PCR reaction for RPGR gene, Forward and Reverse primers for exons;

Orf 15-1R CTT TCC TTC TGA TGG CCC TG
Orf 15-1F CGG TAT GGC AGG AAA TTG ATT G
Orf 15-3R AACACGTAATGAGTGCCCGT
Orf 15-3F GTATCAGGAGACAGGCGAAGAA

Amplicons were screened for mutation with direct DNA sequencing in ABI Prism Genetic Analyzer 310 instrument. Sequence evaluation process were performed using the Sequence Analysis, SeqScape and BioEdit softwares with the mutation tables. Families with a provisional clinical diagnosis of adRP, and a pedigree consistent with XLRP were selected from a cohort of 100 RP.

RESULTS

In our study, 100 individuals who had autosomal recessive inheritance RP and whose clinical diagnosis were approved in Manisa State Hospital Eye Clinic were consisted the study group .100 healthy volunteer individuals who had without RP anamnesis and RP diagnostic report were consisted the control group. DNAs which were isolated with DNA isolation kit from blood samples, were amplified with PCR after then were sequenced with ABI Prism 310 Genetic Analyzer instrument. Exons RPE65 gene ORF15 exon of RPGR gene were selected for the mutation screening. DNA samples were sequenced with ABI Prism 310 Genetic Analyzer instrument and were analyzed with Sequencing Analysis, SeqScape and BioEdit softwares by using the mutation tables

Screening for RPE65 Gene Mutations

RPE65 gene which is expressed in the retinal pigment epithelium containing 14 exons cause to autosomal recessive childhood retinal dystrophy (%2) and Leber congenital amaurosis. We examined 4, 5,10,11 and 13. exons of this gene in 50 unrelated patients with autosomal recessive retinitis pigmentosa (RP), in 50 patients with isolate RP.As a result of the sequence screening in 4 individual patients, , two mutations which were known related to RPE65 gene revealed in BBS. Two brothers were Bardet-Biedl Syndrome consisted

of our patients group. Individual patients were detected. 2 individual patients had Bardet-Biedl syndrome. E352E G> A Glu352Glu (Syn; 1056G> A) and R91Q G> A homozygous mutations were identified.

RPGR and RPE65 genes with di-deoxy sequencing.

Results of pedigrees drawings of mutations in the families of index patients having of genes RP65 (Figure 4-5) and RPGR (Figure 6) tested with di-deoxy sequencing:

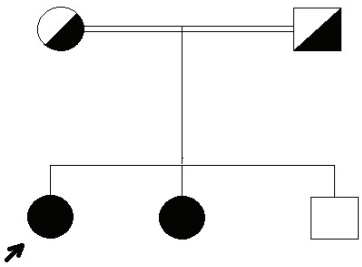

Figure 4. The pedigree of the patient No. 7 who detected the G> A R91Q heterozygote mutation in the exon 4 of the RPE65 genes with di-deoxy sequencing.

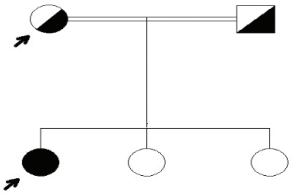

Figure 5. The pedigree of the patient No. 1 and 7 who detected the G>A E352E mutation in the exon10 of the RPE65 genes with di-deoxy sequencing.

Screening for RPGR Gene Mutations

We examined ORF15 exons of this gene in 50 unrelated patients with retinitis pigmentosa (RP), in 50 patients with isolate X-linked Retinitis Pigmentosa .In our study, ORF15+1478T>A heterozygote mutation, ORF15+1643 and ORF15+1677 polymorphisms were detected which were caused X-linked RP and related with RP in RPGR gene. They have found in different communities frequently. Our results has showed compliance with the scanned publications.

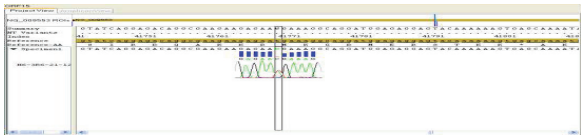

Figure 6. The base change T→A in the ORF15 of the RPGR genes with di-deoxy sequencing.

Statistical Evaluation of Data RPE65 gene and RPGR gene

As a result of our study, G>A R91Q heterozygote mutation in Exon 4 of RPE65 gene in 1 individual patient (% 2), G>A E352E polymorphism in Exon 10 of RPE65 gene in 7 individual patients (% 14) were detected. 36% of 50 individual patients were female, 64% male. 54% of healthy individuals were female, 46% male. As a result of the sequence screening, ORF15+1478T>A heterozygote mutation in ORF15 exon of RPGR gene in 2 women patients (4%), ORF15+1643 and ORF15+1677 polymorphisms in ORF15 exonof RPGR gene in 2 men patients were detected.

DISCUSSION

RP is inherited as an autosomal dominant trait in about 15–20% of families, an autosomal recessive trait in about 20–25% of families, and an X-linked trait in about 10–15% of families, with digenic patterns occurring rarely. In addition, about 50% of RP cases are sporadic, although many of these cases may represent autosomal recessive RP1. X-linked RP (XLRP) is the most severe form of RP in terms of age of onset and progression, with affected males generally showing more severe clinical features than affected females. They usually experience night blindness and loss of dark adaptation in the first decade of life. Peripheral visual fields begin to constrict in the second decade and complete loss of central visual acuity generally occurs in the fourth or fifth decade. In contrast, female carriers show variable clinical symptoms of the disease with visual impairment usually beginning in middle age[3].

Over 40 loci for human hereditary retinal degenerations involving the photoreceptors and retinal pigment epithelium are known (http:yyutsph.sph.uth.tmc.eduywwwyutsphy RetNetyhome.htm). Most of these loci are unidentified genes that have been recognized by linkage studies alone. To explore the possible role that defects in RPE65might play in the etiology of retinal degenerations, we screened a large cohort of unrelated patients with retinitis pigmentosa or Leber congenital amaurosis for mutations. Since RPE65 is particularly visually expressed, in this study, forms not related to syndrome are concentrated on patients. These results have substantive implications for calculation of recurrence risk, genetic counseling, and potential treatment options, and illustrate the importance of screening families with a provisional diagnosis of autosomal inheritance.ORF15 mutations have caused 50-60% of XLRP cases in Europe and North America. Furthermore ORF15 mutations seen in only male patients (simplex male) whose families have not got another patient . Retinitis pigmentosa which is on of the inherited retinal dystrophies leads to night blindness, progressive loss of peripheral visual field and the complete elimination of central vision at the last stage. Performed fundamental scientific studies in our country are very low than abroad with this disease grup that is clinical and genetically highly heterogeneous.It's very important to evaluate hereditery eye diseases in Turkish patients from molecular aspect, to obtain the genetic informations of our society and in order to establish database. Since relatives are marginally widespread in our country, clinicians have a lot of work to prevent Retinitis pigmentosa disease, which has an autosomal recessive transition.It is important to inform their parents about consanguineous marriages and the cause of the disease.

Compared with the literature, our study showed that the RPE65 gene has the same results in terms of the R91Q mutation (pathogenic) at the exon 4 'and the E352E polymorphism (nonpathogenic) at the RPE65 gene Exon 10 (2,6,7,8,9,10,11,12). Our mutation and polymorphism results were found to be lower than previous sequence studies [14]. This is due to the fact that the ORF15 gene is a very long exope and contains repetitive sequence extensions, so we do not have difficulty in optimizing the primers we use in sequencing and we are working with fewer primers (2

primers).There are publications on working with 5 primers [13] . It is stated that 80% of the mutations in XLRP patients occur in the ORF15 region of the juice, but very few mutations were found in the findings.The reason for this is that the number of primers we can optimize and the mutations in the patient group are not ORF15 mutations but other RP2 gene mutations [14]. The other important issue is that family relationships are well defined and the clinical diagnosis must be precisely addressed.Clinicians have a lot of work to do at this point, and as we are in the group of patients involved in this study, we propose that the only male patients in the family should be examined clinically for their mother and sisters.

In conclusion, this study is the first data in selected exons of RPE65 autosomal recessive-inherited and XLRP-inherited RPRG genes with Retinitis pigmentosa patients obtained by DNA sequencing method in Aegean Region in Turkey. Total 100 patients and 100 control individuals were included.In Turkey, identification of selected exons of RPE65 and RPGR gene mutations in Retinitis pigmentosa patients will help to examine the molecular pathogenesis and we hope that our results will guide researchers and clinicians working on this issue in near future to obtain more data in different regions of Turkey.

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