Novel null mutations in the EYS gene are a frequent cause of autosomal recessive retinitis pigmentosa in the Israeli population

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ABSTRACT

Purpose: To characterize the role of EYS, a recently identified retinal disease gene, in families with inherited retinal degenerations in the Israeli and Palestinian populations.

Methods: Clinical and molecular analyses included family history, ocular examination, full-field electroretinography (ERG), perimetry, autozygosity mapping, mutation detection, and estimation of mutation age.

Results: We performed autozygosity mapping in 171 consanguineous Israeli and Palestinian families with inherited retinal degenerations. Large homozygous regions including the EYS gene were identified in 15 of the families. EYS mutation analysis in the 15 index cases, followed by genotyping of specific mutations in additional 121 cases with inherited retinal degenerations, revealed five novel null mutations, two of which are founder mutations, in 10 Israeli and Palestinian families with autosomal recessive retinitis pigmentosa (arRP). The most common mutation we identified was a founder mutation in the Moroccan Jewish sub-population. Using the ESTIAGE program, we estimate that the age of the most recent common ancestor is 26 generations. The retinal phenotype in most patients was a typical yet relatively severe RP, with an early age of onset and non-recordable ERGs upon presentation.

Conclusions: Our results demonstrate that EYS is currently the most commonly mutated arRP gene in the Israeli population, mainly due to founder mutations. EYS mutations were associated with an RP phenotype in all patients, and we predict that the gene plays only a minor role in causing other retinal phenotypes.
INTRODUCTION

Retinitis pigmentosa (RP), with a worldwide prevalence of about 1:3,500,\textsuperscript{1-3} is a group of hereditary degenerative diseases of the retina and is considered one of the most heterogeneous genetic diseases in humans. The disease appears with different modes of inheritance including autosomal recessive (50-60%), autosomal dominant (30-40%), or X-linked (5-15%).\textsuperscript{4} A total of 25 genes have so far been described to cause non-syndromic autosomal recessive RP (arRP) and four additional loci have been identified by linkage studies (see the RetNet database at http://www.sph.uth.tmc.edu/RetNet/home.htm). In 1998, homozygosity mapping led to the identification of the RP25 locus in Spanish families.\textsuperscript{5} Subsequent linkage analyses showed that additional families with arRP from various origins, including Pakistani\textsuperscript{6} and Chinese,\textsuperscript{7} were linked to the RP25 locus. Extensive fine mapping and sequencing efforts in the RP25 region led to a refinement of the linked region,\textsuperscript{8} and eventually to the identification of the causative gene simultaneously by two groups that used different gene hunting strategies.\textsuperscript{9, 10} Abd El-Aziz and co-workers used a multi-step analysis in which they excluded 60 of the 110 genes in the RP25 region, refining the locus to a 2.67 cM region, and identified a large genomic deletion in one of the linked families.\textsuperscript{10} Collin and co-workers, on the other hand, applied homozygosity mapping in non-consanguineous families, using genome-wide single nucleotide polymorphism (SNP) genotyping.\textsuperscript{9} The RP25 gene identified by both studies, termed \textit{EYS} (\textit{eyes shut homolog}), spans almost 2 Mb of genomic sequence and includes 44 exons coding for a 10,475 nucleotide transcript. The gene is abundantly expressed in the human retina\textsuperscript{9, 10} and the protein is localized to the outer segments of the photoreceptor cells.\textsuperscript{10} The retinal function of the EYS protein is still unknown. It is a large protein, composed of 3165 amino acids, containing a signal peptide, 28 EGF-like and 5 laminin A G-like domains. The human EYS protein is a homolog of the \textit{Drosophila} eyes shut (spacemaker) protein, which is an
extracellular matrix protein essential for photoreceptor development and morphology of the insect eye.\textsuperscript{11,12}

Eight \textit{EYS} mutations, all of which are null, have been reported to date, in eight arRP families.\textsuperscript{9,10} Most patients with \textit{EYS} mutations had the clinical diagnosis of RP with an autosomal recessive inheritance pattern,\textsuperscript{9,10} but interestingly, one patient had a cone-rod pattern of photoreceptor loss.\textsuperscript{9}

In a study aimed to estimate the prevalence of RP in the Israeli Jewish population, 341,175 individuals aged 17-20 years were screened for visual acuity problems, and those who had less than 20/25 were further examined by an ophthalmologist.\textsuperscript{13} The prevalence in this study was estimated as 1:4,610.\textsuperscript{13} Since many RP patients in this age group have relatively preserved visual acuities, this is probably an underestimation. The Israeli and Palestinian populations serve as important genetic sources for identifying autosomal recessive disease genes since the Muslim population is enriched with consanguineous marriages and the Jewish population was divided into isolated ethnic groups during history, leading to relatively high inbreeding levels. A study conducted in schools for the blind in the West Bank and Gaza in 1992 concluded that 44-85\% of the affected children (depending on location) were the offspring of a consanguineous marriage.\textsuperscript{14} Only limited information is currently available on the genetic causes of non-syndromic RP in the Israeli and Palestinian populations, with only a few mutations reported thus far in the \textit{CERKL}, \textit{CRB1}, \textit{NR2E3}, \textit{RDH12}, \textit{TULP1}, and \textit{USH2A} genes.\textsuperscript{15-19}

As part of our genetic analysis of Israeli and Palestinian families with autosomal recessive retinal degenerations, we used autozygosity mapping as the major mapping tool and identified families with arRP linked to the \textit{EYS} region. Sequence analysis revealed five novel null mutations, two of which were founder mutations, in 10 families with arRP. The phenotype of most affected individuals was typical for arRP although some clinical variation was evident.
**METHODS**

**Patient recruitment:** The tenets of the Declaration of Helsinki were followed and informed consent was obtained from all patients who participated in this study prior to donation of a blood sample. Blood samples for DNA analysis were obtained from the index patient as well as other affected and unaffected family members.

**Genetic analysis:** Genomic DNA was extracted from peripheral blood samples of index cases and their family members using the FlexiGene DNA kit (QIAGEN). Whole genome SNP analysis was performed using the Affymetrix GeneChip Human Mapping 10K Xba 142 2.0 microarray. Primers for the screening of EYS by PCR amplification have been reported previously.9 PCR was performed in a volume of 25 µl reaction with 35 cycles. Mutation analysis was performed by direct sequencing of purified PCR products.

Genotyping of specific mutations was performed as follows: Two mutations, c.403delA,406G>T,410_424del15 (exon 4) and c.4361_4362CC>AG (exon 26) were genotyped using the restriction enzymes DdeI and MnlI, respectively. For the genotyping assay of the c.3715G>T (exon 25) mutation, we designed allele-specific primers to distinguish between the mutant and normal alleles (primer sequences available on request) and used the NlaIII enzyme for restriction analysis. The c.1211_1212insA (exon 8) and c.8218_8219delCA (exon 43) mutations were screened by sequencing analysis. The possible effect of sequence changes on the splicing of the corresponding exon was estimated using Splice Site Prediction by Neural Network at http://www.fruitfly.org/seq_tools/splice.html.

**Estimation of mutation age:** We used the ESTIAGE program20 to calculate the age (in generations) of the most recent common ancestor of patients carrying the c.403delA,406G>T,410_424del15 mutation. ESTIAGE is a likelihood-based method which uses multi locus marker data from patients carrying the same mutation, assuming they descended from a common ancestor who introduced the mutation. An estimate of the
number of generations since the most recent common ancestor is obtained from the size of the haplotype shared by the individuals on each side of the disease locus. The method uses the haplotype information in patients carrying the mutation and in controls to identify the most likely positions of recombination events on the ancestral haplotype. This method was reported to be efficient for a very small number of affected individuals in rare diseases. We used SNP genotyping data from 36 markers on eight chromosomes carrying the mutation and 46 population-matched control chromosomes. Allele frequencies were calculated based on the control population.

Clinical evaluation: Complete ophthalmic examinations were performed including refraction, visual acuity, biomicroscopic slit lamp examination, funduscropy. Retinal imaging included color fundus photographs, optical coherence tomography (OCT) and autofluorescence. Best corrected visual acuity was measured in each eye separately using an ETDRS chart, and presented as a decimal ratio. In patients with visual acuity lower than 0.05, the distance at which fingers could be reliably counted was recorded. Retinal function was evaluated according to the level of patient cooperation by full-field electroretinography (ERG), Goldmann kinetic and/or Humphrey static Perimetry as previously described. Briefly, full-field ERGs were recorded using corneal electrodes and a computerized system (UTAS 3000, LKC, MD). In the dark-adapted state, rod responses to a dim blue flash (Wratten 47b) and mixed cone-rod responses to a white flash (2.35 cd·sec/m2) were acquired. Cone responses to 30 Hz flashes of white light (9.4 cd·sec/m2) were acquired under a background light of 21 cd/m2. All ERG responses were filtered at 0.3-500 Hz and signal averaging used.
RESULTS

Autozygosity mapping and EYS mutation analysis

We recruited 712 Israeli and Palestinian families with hereditary non-syndromic retinal disease, 307 (including 121 with RP and 43 with Leber congenital amaurosis [LCA]) of which were consanguineous. Aiming to identify the causative gene in the consanguineous families, we used the 10K Affymetrix SNP microarray system for autozygosity mapping in patients from 171 of the families (82 with RP, 28 with LCA, and 61 with cone-dominated diseases). Autozygosity analysis revealed 18 patients from 15 different families (Table 1) who had a large homozygous region harboring the recently identified EYS gene and fulfilled two criteria: the genetic size of the homozygous region was at least 10 cM and it was among the 10 largest homozygous regions in the affected individual (Table 1).

Interestingly, patients from three of the families (MOL0318, MOL0501, MOL0662- Fig. 1) were of Moroccan Jewish ancestry and shared a large region covered by 44 consecutive homozygous SNP markers. Sequence analysis of the 44 EYS exons and exon-intron boundaries in the 15 above-mentioned index cases revealed two novel homozygous null mutations in 4 families (Table 1 and Fig. 2). Patients from the three above-mentioned Moroccan Jewish families were homozygous for a novel complex frameshift mutation c.403delA,406G>T,410_424del15 (hereafter referred to as p.Thr135LeufsX25) in exon 4, which causes a premature stop codon at position 160 due to a single base pair deletion followed by a 15-bp deletion (Fig. 2A). In addition, an isolated case from a consanguineous Palestinian Muslim family (MOL0410; Fig. 1 and Table 1) was homozygous for a novel nonsense mutation, c.4361_4362CC>AG (p.Ser1454X), in exon 26 of the EYS gene (Fig. 2D). Aiming to assess the frequency of these mutations among patients from the corresponding sub-populations, we screened a set of 56 Jewish families with RP from North-African ancestry (from Morocco, Libya, Tunisia, and Algeria) for the
p.Thr135LeufsX25 mutation and 47 Muslim families with RP for the p.Ser1454X mutation. The analysis revealed four additional index patients who were either heterozygous (TB59 III:1 and MOL0626 III:1; Fig. 1 and Table 1) or homozygous (MOL0792 II:2 and MOL0806 III:7) for the p.Thr135LeufsX25 mutation.

The two heterozygous patients were from non-consanguineous families, and we therefore assumed that the patients are compound heterozygous. Screening of the EYS gene indeed revealed novel heterozygous null mutations in these patients. Patient MOL0626 III:1 (from Moroccan Jewish ancestry) was heterozygous for a frameshift mutation, c.1211_1212insA (p.Asn404LysfsX2; Table 1 and Fig. 2B), in exon 8. No additional family members were available to confirm the compound heterozygous state. Aiming to evaluate the frequency of the c.1211_1212insA mutation in North African Jewish RP patients, we sequenced exon 8 in 47 additional index RP patients, but none of them carried the c.1211_1212insA mutation or any other mutation in this exon. Patient TB59 III:1 was heterozygous for a novel frameshift mutation, c.8218_8219delCA (p.His2740TyrfsX27; Fig. 2E), in exon 43. Cosegregation analysis in family TB59 (Fig. 1) revealed that the p.Thr135LeufsX25 mutation was on the maternal allele (of Moroccan Jewish ancestry) and the c.8218_8219delCA mutation was on the paternal allele (of Iraqi Jewish ancestry). We therefore evaluated the frequency of the c.8218_8219delCA mutation in 31 Oriental Jewish RP patients (mainly from Iran, Iraq, and Afghanistan) and identified two non-consanguineous patients from Iraqi Jewish origin who were either homozygous (MOL0640 II:1) or heterozygous (TB24 III:2) for the mutation. All the alleles carrying the c.8218_8219delCA mutation were from an Iraqi Jewish origin and shared an identical haplotype within EYS, indicating a founder mutation. Sequencing analysis of the remaining exons in TB24 III:2 revealed yet another novel nonsense mutation, c.3715G>T (p.Glu1239X), in exon 25 (Table 1 and Fig. 2C). The mutation could not be identified in any of 31 additional RP patients from Oriental Jewish origin.
In addition, we identified 29 sequence changes in our genetic screen, of which 14 were nonsynonymous changes which are known polymorphisms in the SNP database (Supplementary Table). None of these sequence changes is likely to be pathogenic.

**Haplotype analysis and estimation of mutation age**

The most common EYS mutation identified in this study was the p.Thr135LeufsX25 mutation identified in 7 RP families, all of Moroccan Jewish ancestry, indicating a founder effect. Screening of normal individuals of Moroccan origin revealed that one out of 94 control individuals was heterozygous. Haplotype analysis of four patients who were homozygous for this mutation revealed a shared homozygous region composed of nine SNP markers covering 3.2 Mb or 2.1 cM (Supplementary Fig. 1). The shared homozygous region in three of these patients from consanguineous families is much larger, harboring 44 markers along a region of 17 Mb (6.3 cM). Two pieces of evidence suggest that the mutation is relatively young: the relatively large size of the shared homozygous region and the fact that the mutation was found only in Moroccan Jews and not in Jews originating from neighbouring countries (Algeria, Libya, and Tunisia). Aiming to estimate the mutation age, we used genotyping data from 36 SNP markers (8 within the EYS gene and 28 flanking it) on eight chromosomes carrying the mutation (MOL0318 II:13, MOL0318 III:2, MOL0501 II:1, and MOL0662 IV:1) and 46 population-matched control chromosomes. The data set was analyzed using the ESTIAGE software (see Methods) and the number of generations since the most recent common ancestor was estimated as 26 (95% CI of 12-56 generations) or 650 years (based on a mean generation time of 25 years).

**Ocular phenotype associated with EYS mutations**

We clinically examined 15 patients with EYS mutations and performed ERG testing in 11 (Table 2). All patients with EYS mutations showed characteristic manifestations of RP, with
a relatively severe course of disease. Night vision, visual fields, and ERG responses were markedly impaired already at early ages. Both myopic and hyperopic refractive errors were present. Fundus appearance was typical for RP including bone spicule-like pigmentation, pallor of the optic discs, and narrowing of blood vessels (representative fundus mosaics of the left eyes of patient TB24 III:2 at age 63 and patient MOL0501 II:3 at age 52 are shown in Fig. 3A and 3E, respectively). Macular involvement is evident also on autofluorescence imaging (Fig. 3B,F) and OCT, with retinal thinning and atrophy (Fig. 3D,H). Visual fields in these two patients are markedly constricted (Fig. 3C,G). In almost all patients for whom ERG data were available (ages between 19-51 years), both scotopic and photopic full-field ERG responses were extinguished, indicating severe retinal involvement (Table 2). Only in one patient (MOL0640 III:1) scotopic as well as photopic ERG responses were measurable, although also severely reduced. This patient initially presented with sector RP at the age of 25 (based on funduscopic and visual field findings) which later progressed to widespread, generalized retinal involvement. The mean 30 Hz cone flicker ERG amplitude measured at our center on the first ERG test performed in each patient was significantly lower in the EYS group as compared to pooled, first test-ERG amplitudes in RP patients in our population (Fig. 4). The EYS group included 11 patients with a mean age of 34 years and mean ± SD amplitude of 0.65 µV ± 2.17. The control RP group included 240 patients with a mean age of 29.8 years and mean ± SD amplitude of 17.7 µV ± 22.2 (p-value for cone flicker ERG amplitude difference < 0.01).

DISCUSSION

EYS represents a major arRP gene, as can be appreciated from the widespread ethnic origins of families with arRP due to EYS mutations thus far reported. In the present study, we show that mutations in EYS are currently the most frequent cause of non-syndromic arRP in the Israeli population, mainly due to founder mutations in two ethnic groups: Moroccan and
Oriental Jews. The five novel EYS mutations reported here account for at least 7% of arRP families (10 out of 141 families) we have thus far recruited from the Israeli and Palestinian populations. The most common mutation we identified in this study (p.Thr135LeufsX25) is a founder mutation in the Moroccan Jewish population accounting for about 19% (12 out of 64 chromosomes) of non-syndromic arRP alleles in this population, and is therefore currently the most common cause of RP in Jews of Moroccan ancestry.

Similar to previous studies, most of our patients with EYS mutations manifested typical and rather severe arRP. One exception is an isolated case who initially presented with a milder phenotype, sector RP, and was homozygous for a null mutation in exon 43. Although one might expect that the nonsense-mediated mRNA decay (NMD) mechanism would prevent protein production of this mutant allele, it is tempting to speculate that some transcripts will escape degradation, as reported in many other cases (see Holbrook et al.\textsuperscript{22} for review), manifesting a milder, more localized, phenotype. Mutations that are likely to result in a similar effect, however, were reported to cause the typical widespread and severe RP phenotype.\textsuperscript{9} Only one patient to date was reported to manifest a different retinal phenotype, cone-rod dystrophy (CRD), due to a null mutation located at the very end of the carboxy-terminus of EYS, thereby resulting in the absence of only the last 10 amino acids. His older sib, though, displayed RP even suggesting the possibility of variable intrafamilial expression of certain mutations,\textsuperscript{9} probably due to other factors influencing clinical expression. We included 61 families with AR cone dominated diseases (mainly CRD) in our autozygosity analysis, but only one (in which no EYS mutations were identified) was homozygous at the EYS locus, indicating that mutations in EYS are a rare cause of CRD in our population. The clinical variability in patients with null mutations in EYS suggests that other factors contribute to the severity of disease in these cases, as previously suggested.\textsuperscript{9}

It is interesting to note that all EYS mutations thus far reported,\textsuperscript{9,10} as well as the five novel mutations reported here, are null mutations (either frameshift or nonsense). A
large number of *EYS* missense changes are known (some of which appear as entries at dbSNP), but none of which is currently considered as a cause of disease. Such missense changes, in combination with a null mutation on the counter allele, might result in a milder retinal phenotype. Another possible explanation for the lack of pathogenic missense mutations in *EYS* is the ability of the protein to tolerate single amino-acid alterations, affecting only a specific protein domain, due to the multiple functional domains (e.g. EGF-like and laminin A G-like domains) in the EYS protein. This phenomenon resembles the mutation spectrum of well-studied retinal genes, including *CHM* and the *RPGR-ORF15* terminal exon, in which only null mutations have been reported so far. Such genes will therefore cause a retinal phenotype only if no protein is generated. One can also assume that some of the polymorphic missense changes might result in an expressed protein with reduced activity. This makes *EYS* an attractive candidate for modulating retinal disease severity in cases caused by mutations in other retinal genes, mainly those encoding proteins that interact with EYS, such as *PROM1*. Since *EYS* was only recently identified, further *EYS* mutation analyses are likely to result in a more accurate and comprehensive *EYS* mutation spectrum.

The most common mutation we identified here (p.Thr135LeufsX25) is a founder mutation in the Moroccan Jewish sub-population. The history of the Jewish population in North Africa (including Morocco, Algeria, Libya, and Tunisia) is ancient and complicated. The population was founded about 2,000-2,600 years ago and since then underwent a number of historic events that had a dramatic effect on the population size, including a few waves of immigrations (during the first temple period about 600 years BCE and in 1492 due to the expulsion from Spain), the conversion of Berber tribes to Judaism (during the sixth and seventh centuries), and the persecution of a major part of the community in 1033 and 1232. By 1948, the Jewish Moroccan population (the major North African Jewish population) was estimated to contain 270,000 individuals, most of whom immigrated to
Israel once the state was established in 1948. Using the ESTIAGE program, which was designed for rare diseases, we estimated the age of the most recent common ancestor to be relatively young, 26 generations (or 650 years) ago. This young age is supported by the fact that the mutation was found in Jews originated from Morocco (and not other North African countries), and by the relatively large size of the shared homozygous region in most of the studied patients. Up to date, the estimated ages of only two founder mutations in the Moroccan Jewish population have been reported and found to be relatively ancient (2,600-2,700 years ago).\textsuperscript{27,28}

In summary, in this study we report the mutation spectrum of $EYS$ in a cohort of Israeli and Palestinian RP patients which includes five novel mutations and show that $EYS$ is the most frequently mutated arRP gene currently known in the Israeli population.
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REFERENCES


FIGURE LEGENDS

FIGURE 1: Israeli and Palestinian families with EYS mutations. Numbers above the family trees indicate the family serial number, numbers within symbols represent the number of siblings, numbers below symbols indicate the generation and individual numbers, arrows represent index cases, and a double horizontal line designates consanguinity with an indication of the consanguinity level (e.g. 2:2 designates first cousin marriages). For each recruited individual, the EYS genotype is depicted below the individual symbol. M1- p.Thr135LeufsX25, M2- p.Ser1454X, M3- p.Asn404LysfsX2, M4- p.His2740TyrfsX27, M5- p.Glu1239X.

FIGURE 2: The chromatograms of the five novel EYS mutations. For each mutation, the wild-type (wt) sequence is depicted with the homozygous or heterozygous sequences when available. The mutation location is indicated by either an arrow (for a nucleotide change) or a horizontal line (for a sequence change affecting more than a single nucleotide). A- c.403delA,406G>T,410_424del15; p.Thr135LeufsX25 (exon 4), B- c.1211_1212insA; p.Asn404LysfsX2 (exon 8), C- c.3715G>T; p.Glu1239X (exon 25), D- c.4361_4362CC>AG; p.Ser1454X (exon 26), E- c.8218_8219delCA; p.His2740TyrfsX27 (exon 43).

FIGURE 3: Fundus imaging and visual fields. Representative fundus images and Goldman visual fields of the left eyes of patient TB24 III:2 (panels A-D) at age 63 and patient MOL0501 II:3 (panels E-H) at age 52 are shown. The typical funduscopic findings of RP are present, including bone spicule-like pigmentation, pallor of the optic discs, and narrowing of blood vessels (A,E). Macular involvement is evident also on autofluorescence
imaging (B,F) and OCT, with retinal thinning and atrophy (D,H). Note more severe foveal atrophy in patient MOL0501 II:3 which correlates with the lower visual acuity of this patient (Table 2). Visual fields are markedly constricted in both patients (C,G).

FIGURE 4: A graph representing cone 30-Hz ERG amplitude (Y-axis) versus the age (X-axis) at which ERG data was first obtained from 11 patients with EYS mutations (filled diamonds) versus 240 patients diagnosed with RP (open squares). Each data point represents the average cone flicker amplitude of the two eyes of each patient.
Table 1: Data regarding patients screened for mutations in the EYS gene

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<th>Rank among homozygous regions (based on genetic size)</th>
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* In bold are patients in whom EYS mutations were identified. The corresponding family trees are shown in Fig. 1.

† arRP, autosomal recessive retinitis pigmentosa; sRP, simplex retinitis pigmentosa; sSTGD, simplex Stargardt disease; sLCA, simplex Leber’s congenital amaurosis.

‡ Level of consanguinity is measured by the number of generations separating the spouse from the common ancestor (e.g. 2:2 designates first cousins, 2:1 designates marriage between an uncle and his niece).

§ Data are based on 10K SNP analysis (see Materials and Methods).

‖ The predicted effect of the mutation on the protein sequence is presented. Please see Supplementary Table for additional information.

n.d.- not done.
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*FFERG=Full field electroretinogram including the followings: Rod response b-wave amplitude (in μV, normal > 200μV); Mixed cone-rod a/b wave (in μV, normal a-wave > 90μV, normal b-wave > 400μV); Cone response (in μV, normal > 60μV), implicit time (in msec, normal ≤ 33 msec).

CF, counting fingers; F, female; M, male; m, meter; NA, Not available; NP, not performed.

First line (2nd-6th column) indicates the right eye, second line indicates the left eye.
FIGURE 2

A

WT

Mutant

Homogygote

Heterozygote

c.406
c.403  
c.410-424

c.406G>T

c.403G>A

c.410-424del15

B

WT

Mutant

Heterozygote

c.1211_1212

c.1211_1212insA

C

WT

Mutant

Homogygote

Heterozygote

c.3715

c.3715G>T

D

WT

Mutant

Homogygote

Heterozygote

c.4361_4362

c.4361_4362insCC>AG

E

WT

Mutant

Homogygote

Heterozygote

c.8218_8219

c.8218_8219delGA
FIGURE 3

A

B

C

D

E

F

G

H