

A Two-stage Approach Identifies a Q344X Mutation in the Rhodopsin Gene of a Chinese Singaporean Family with Autosomal Dominant Retinitis Pigmentosa

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Abstract

Introduction: Retinitis pigmentosa (RP) is a group of hereditary retinal diseases in which photoreceptor cells degenerate. It is both clinically and genetically heterogeneous. Using a two-stage approach by combining linkage analysis with mutation detection, we have rapidly identified the gene locus and the mutation site of a Chinese Singaporean family with autosomal dominant RP. **Materials and Methods:** Three Chinese Singaporean families were tested. One family showed autosomal dominant inheritance pattern, while the other two could be recessive or sporadic. Twelve di-nucleotide markers tightly linked to 6 genes known to be responsible for either autosomal dominant or recessive RP were selected for linkage analysis. Cosegregation of marker and disease inheritance pattern permits identification of the target candidate gene. RFLP (restriction fragment length polymorphism) markers were added to confirm the linkage result prior to the detailed mutation detection study. **Results:** With this two-stage strategy, the autosomal dominant RP family showed the rhodopsin locus segregating concordantly with the disease. Mutation screening later identified a nonsense mutation 5261C>T in the last exon of rhodopsin gene. It predicted a Q344X changes at the C-terminus of the gene product, truncating it by 5 amino acids. **Conclusion:** This systematic approach facilitates molecular diagnosis of a genetically heterogeneous disease like RP. This is the first report of an RP mutation in Singapore. This 5261C>T mutation has been reported in the Caucasian, but not the Chinese population. The relatively milder phenotype in this family showed similarity to the reported US family, indicating the correlation of mutation site to severity of disease regardless of ethnicity.

Ann Acad Med Singapore 2005;34:94-9

Key words: Genetic linkage analysis, Mutational analysis

Introduction

Retinitis pigmentosa (RP, MIM#26800) is a group of hereditary retinal diseases that feature progressive photoreceptor degeneration, eventually leading to partial or complete blindness. It is characterised by night blindness and constricted visual field caused by the early loss of peripheral photoreceptors, primarily rods. Central vision could also be lost as the disease progresses. It classically shows fundus changes such as intraretinal pigmentation, narrowed arterioles and optic disc pallor. RP is a common form of inherited blindness worldwide. Epidemiological studies around the world consistently report a frequency of approximately 1 in 4000 to 5000 without apparent ethnic and racial distinctions.^{1,2}

RP is clinically and genetically heterogeneous. The modes of inheritance documented include autosomal dominant (adRP), autosomal recessive (arRP), X-linked (xLRP) and the rare digenic form. In the western countries, it was reported that adRP represents 15% to 20% of all RP cases; arRP, 20% to 25%; xLRP, 10% to 15%, with sporadic cases constituting the remaining 40% to 55%.³ However, these frequencies vary in different populations throughout the world.^{4,5} A Chinese study reported a prevalence rate of RP in China of 1:3784 and the relative frequencies of the various genetic forms were 5% adRP, 25% arRP, 3% xLRP and 67% sporadic.⁶ The prevalence and relative frequencies of RP in Singapore or the Southeast Asian region are uncertain. A rough estimate based on 26 index cases

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collected at the National University Hospital, Singapore (NUH) indicates that the representations for adRP, arRP, xLRP and sporadic cases are 30%, 8%, 8% and 54%, respectively (CKL Chee, personal communication). Most cases are classified as sporadic as they are simplex cases with no family history and no consanguinity. Some of the sporadic cases could be arRP.

To date, genetic studies have identified at least 38 non-overlapping chromosomal loci implicated in the aetiology of RP, with mutation identified in 31 genes that cause some forms of non-syndromic RP. Of these identified genes, 13 are now known to cause adRP, 17 for arRP and 2 for xLRP (OMIM: <http://www.ncbi.nlm.nih.gov>; RetNet: <http://www.sph.uth.tmc.edu/Retnet>). Most of these genes were mapped or identified through linkage studies or candidate genes screening based on the gene product biological function. Many of these genes encode proteins that participate in biochemical pathways that are specific to the retina. These pathways include the phototransduction pathway and the vitamin A cycle. Other functional categories represented by these RP genes include transcription factors, mRNA splicing factors and structural proteins. The functions of some of these RP gene products are currently unknown.⁷

The extensive genetic heterogeneity of RP renders its molecular diagnosis very difficult. For an assortment of 3 RP families collected in Singapore, we adopted a two-stage approach to screen and identify the underlying mutated gene. First, the involvement of a possible candidate gene was investigated by linkage analysis using short tandem repeat (STR) and restriction fragment length polymorphism (RFLP) markers. Then, any implicated gene was followed up and the mutated site identified by direct sequencing. Six candidate genes were investigated in this study. The selected candidate genes included rhodopsin (RHO), phosphodiesterase α and β subunits (PDEA and PDEB), cGMP-gated channel α subunit (CNCG1), peripherin/rds (RDS) and rod outer membrane 1 (ROM1). Most mutations in RHO and RDS genes have been reported to be associated with adRP, while a few have been linked to arRP (HGMD: <http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html>; <http://www.retina-international.org/sci-news/mutation.htm>). Several studies have estimated that rhodopsin mutations accounted for approximately 25% to 50% of all adRP cases in the USA and the UK.⁸⁻¹⁰ Mutations in PDEA, PDEB and CNCG1 were associated with arRP (Mutview: <http://mutview.dmb.med.keio.ac.jp>), while ROM1 and RDS have been shown to be responsible for digenic RP.¹¹ This two-stage strategy has resulted in the rapid identification of a nonsense mutation of 5261C>T at the exon 5 of RHO gene in one of the local Chinese RP families.

Materials and Methods

Patients and their family members were ascertained at the Department of Ophthalmology, NUH. 5 mL of venous blood was collected for genomic DNA extraction. All 3 families are of Chinese ethnicity. The first family consists of 11 members and 3 generations presenting in autosomal dominant mode of inheritance. The other 2 families each consist of 5 members and 2 generations. The latter 2 families belonged to simplex cases and hence the inheritance could be recessive or sporadic.

Two STR markers were selected to represent each candidate gene. The genetic distance of each STR marker to its candidate gene was obtained from Genethon genetic and National Center for Biotechnology Information (NCBI) integrated maps.^{12,13} All markers are flanking markers of their respective genes, while markers rho and rds are intragenic to RHO and RDS genes, respectively.¹⁴ STR primer sequences were either obtained from the Genome Database (<http://gdb.org>) or were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) based on genomic sequences in Genbank. The primers were designed such that all their PCR products could be pooled and multiplexed as a single electrophoresis run. Table 1 lists the markers and the candidate genes they represented. Marker primers were labelled with fluorescent dye and genotyping was done by multiplexing on an ABI 377 sequencer (Applied Biosystem, Foster City, CA, USA), with allele called by the software

Table 1. Candidate Genes and Selected STR Markers for Genotyping

Candidate gene	Locus	Markers
Rhodopsin (RHO)	3q21-q24	RHO
		D3S3606
Rod cGMP phosphodiesterase subunit (PDEA)	5q31.2-q34	D5S2013
		D5S434
Rod cGMP phosphodiesterase subunit (PDEB)	4p16.3	D4S227
		D4S3038
Rod cGMP gated channel subunit (CNCG1)	4p14-q13	D4S3002
		D4S1536
Peripherin/rds (RDS)	6p	RDS
		D6S1582
Rod outer membrane protein 1 (ROM1)	11q13	D11S480
		D11S4076

STR: short tandem repeat

Genescan v3.1. Linkage analysis was carried out with the computer programme, GAS v2.0 (Alan Young, <http://users.ox.ac.uk/~ayoung/gas.html>).

Rhodopsin (RHO, MIM#180380) polymorphisms, 269A>G, 5145G>A and 5510A>G were adapted as RFLP markers.^{15,16} Primers to the first 4 exons of the rhodopsin gene (Genbank: U49742) were adopted from Dryja et al,¹⁷ while primers for exon 5 were redesigned to include additional flanking intron sequences in order to include the 2 polymorphic sites 5145G>A and 5510A>G. Polymerase chain reaction (PCR) product of rhodopsin gene exon 1 was digested with restriction enzyme Sac II for the RFLP 269A>G, while product of exon 5 were digested with Hinf I or Ava I for RFLPs 5145G>A and 5510A>G, respectively. Digested fragments were analysed on a 2% agarose gel.

Sequencing was carried out on all the 5 exons of the rhodopsin gene. Cycle sequencing was performed with ABI Prism dRhodamine Terminator Ready Reaction Mix and was analysed on the ABI 377 sequencer.

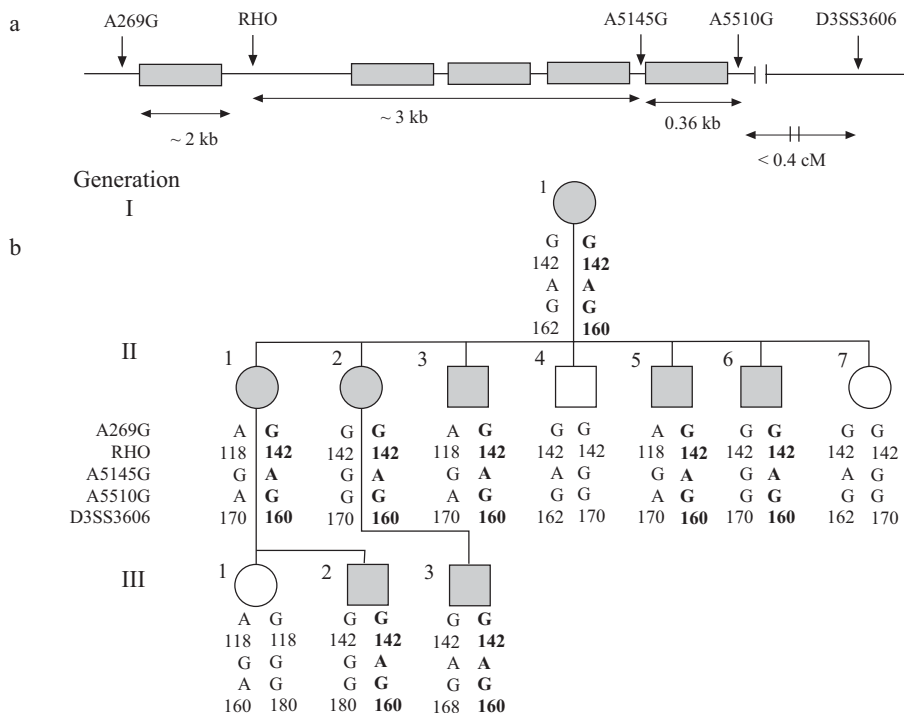
Results

Two-point linkage analysis using an autosomal dominant model for the adRP Singaporean family generated a lod score of 2.39 for the rhodopsin-flanking marker D3S3606.

This marker was estimated to be less than 0.4 cM away from rhodopsin gene. With the recent availability of the human genome map, it is known to be 800 kb away. The intragenic marker RHO, with a heterozygosity of 33%, was not sufficiently informative in this family and produced a lod score of 0.6. Nevertheless, the haplotype formed by these 2 markers produced an inheritance pattern that implicated the involvement of the rhodopsin locus in this adRP family.

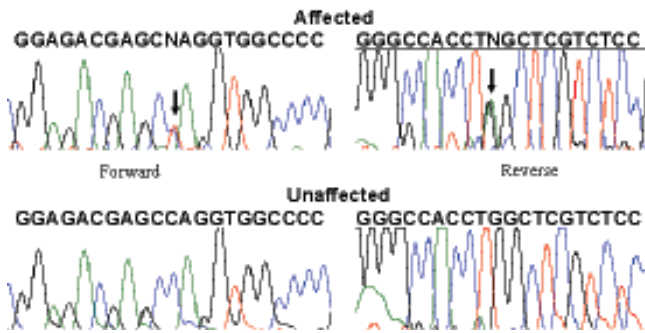
The above positive linkage to rhodopsin locus was further verified by the results of the 3 intragenic RFLP markers. The estimated position of each marker in relation to the rhodopsin gene is shown in Figure 1a. The disease allele has a haplotype **G-142-A-G-160** and it cosegregated with the disease phenotype in this family in an autosomal dominant pattern (Fig. 1b). No markers showed positive linkage with the other 2 families with suspected arRP or sporadic RP.

Direct sequencing of the rhodopsin gene for affected family members in the first family revealed a nonsense C to T substitution at position 5261 in exon 5 (Fig. 2). This heterozygote mutation was confirmed by reverse sequencing and predicted to result in a Q344X mutation, truncating the



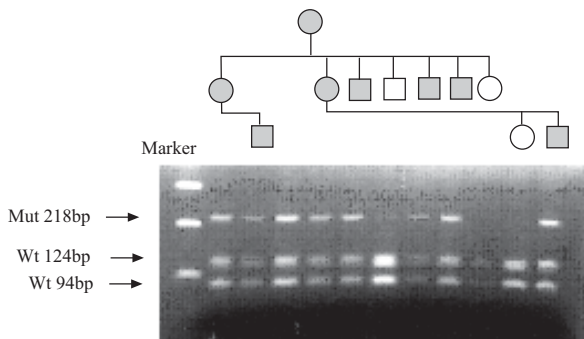
Square: Male; Circle: Female; Filled symbols: individuals with diagnosed retinitis pigmentosa; Bold letter: presumed mutation-carrying haplotype which is shared among affected individuals.

Fig. 1a. Schematic representation of linkage map and markers used in the rhodopsin locus. Fig. 1b. Haplotype distribution at the rhodopsin locus for the Singaporean family with adRP.



Both forward and reverse sequences of affected and unaffected family members are shown. Arrow indicates the mutation site.

Fig. 2. Sequence analysis of rhodopsin gene Q344X mutation.



Each gel lane corresponds to an aligned individual from the Singapore pedigree with adRP. Unaffected individuals do not have the 218 bp fragment.

Fig. 3. Rapid diagnosis of Q344X mutation with BstNI.

last 5 amino acids at the carboxyl terminal of the opsin protein. This heterozygote mutation was found in all affected members of this family but not in any of the normal individuals.

This C to T substitution abolished a BstN I restriction site, generating an extra mutant fragment of size 218 bp on agarose gel. This demonstrated the development of a PCR-RFLP assay as a rapid diagnostic method for this family (Fig. 3).

Discussion

This two-stage approach allows a quick and broad screen of multiple candidate genes, before the laborious mutation screening effort is carried out on the narrowed targets. This approach taps into the advantage of linkage analysis, which could efficiently exclude non-associated loci, hence reducing subsequent mutation screening efforts like sequencing. Furthermore, where there is sufficient linkage data yielding a statistically significant LOD score, the disease locus can be positively identified by linkage alone. Direct mutation screening methods, such as direct

sequencing or sequence analysis with SSCP (single strand conformation polymorphism) or dHPLC (denaturing high performance liquid chromatography), could potentially miss some mutations such as those situated at regulatory and intronic regions, or large heterozygote deletion.

However, this approach also has its inherent weakness. One major limitation is that linkage analysis requires family pedigrees of reasonable size. This limitation was reflected in the 2 small families with simplex cases, where linkage analysis of all markers proved to be inconclusive. It was difficult to include or exclude any of the candidate genes when their inheritance mode had not been established. The second limitation of linkage analysis is the availability of informative markers, both in terms of not recombining with the disease mutation in the studied gene and the marker's informative content. This was reflected in this study by the marker RHO, which although physically located within the rhodopsin gene, failed to produce a positive linkage lod score due to its low heterozygosity content. However, finding informative DNA markers is currently less of a problem, with the availability of whole human genome sequences and polymorphisms in the public domain. It is possible to search through the genome sequence for suitable STR markers that are very near and tightly linked to the query gene.

Nevertheless, regardless of these limitations, in view of the extensive genetic heterogeneity of all forms of RP, this two-stage approach remains a cost-effective and efficient method toward identifying the underlying causative gene mutation, as demonstrated by this rapid identification of the rhodopsin gene mutation in a local RP family. Further improvement to this method could be to construct a specialised marker panel for each specific form of RP. For example, the adRP panel could contain 26 STR markers, 2 per each of the 13 candidate genes known to cause adRP, arranged in multiplex PCR format for rapid screening of adRP family.

The quick RFLP diagnostic method developed for the Q344X mutation provides a rapid means of pre-symptomatic diagnosis for this family. It could help in confirming clinical diagnosis, and should be particularly useful for predictive testing for other younger members who are below the maximum age of presentation, which is the third decade of life. The pre-symptomatic information may be useful for genetic counselling in terms of vocation selection or lifestyle modification since this family has shown a late-onset phenotype presentation.

This Q344X mutation is the first reported case of a rhodopsin gene mutation in the Singaporean population. A similar mutation has been reported in 1 US family and a single case has been reported in Germany.^{18,19} The 3' end of the rhodopsin gene is a common mutation region with

several mutation sites, including some common mutation sites reported in separate geographical populations (HGMD: <http://archive.uwcm.ac.uk/uwcm/mg/ns/1/120347.html>; <http://www.retina-international.com/sci-news/rhomet.htm>). Interestingly, to date, all the reported rhodopsin mutations in Asia have been concentrated at the carboxyl end. P347L and 5211delC were identified in Hong Kong Chinese,²⁰ while P347L, E341X and V345M were reported in Japan, China and India, respectively.²¹⁻²³ P347L has been identified worldwide, with reports from the USA, UK, Germany, South Africa, Spain and Lithuania.²⁴⁻²⁶ 5211delC has also been reported in an Italian family,²⁷ while V345M has been found in the USA.¹⁷

The clustering of mutation sites at the C-terminal end of the rhodopsin gene in different populations supports the functional importance of the C-terminal region to the opsin protein. It had been demonstrated that transgenic mice harbouring the Q344X mutant showed defective localisation of the mutant protein to the inner segment plasma membrane.²⁸ The role of rhodopsin's C-terminal in protein transport was finally clarified with the discovery that the wild-type, but not mutated C-terminus, binds to the Tctex-1 protein, a dynein light chain. It was suggested that Tctex-1 binds to rhodopsin via its C-terminal and guides the transport of rhodopsin to the proper destination at the rod outer segment.²⁹ Other workers also provided evidence that the loss of the highly conserved C-terminal sequence QVAPA of the opsin protein would misdirect the protein to incorrect cellular location, finally resulting in rod cell death.^{30,31}

This Singaporean Chinese family with rhodopsin Q344X mutation generally presented with a mild phenotype. Older members of the first and second generations reported that they first experienced nyctalopia in their 20s or older. Some affected members at 40 years old still retain visual acuity at 6/24. The oldest member, at over 70 years of age, has visual acuity of 6/60 in each eye. One affected third generation member (Individual III-3, Fig. 1b) was seen in 1990 at the age of 8. At that time he had vision of 6/7.5 in each eye, no nyctalopia and only subtle retinal pigmentary change which could have been within normal limits. Goldmann visual fields were normal. Eight years later at 16 years of age, he had some difficulty gauging distances at night, but did not bump into objects. Humphrey 24-2 visual fields done at that time showed generalised relative peripheral constriction of up to 10 degrees of fixation. The retinal pigment epithelium showed slightly greater pigmentation, but there was no intraretinal pigment migration, narrowed arterioles or optic disc pallor. Four years later, at 20 years of age, there was no detectable deterioration. His affected cousin (Individual III-2, Fig. 1b) was found to have the rhodopsin mutation in 1999, and was first seen 2 years later when he

was 19 years old. He gave a history of prolonged dark adaptation. His vision was 6/7.5 in both eyes. There were no obvious retinal signs of RP, only mild pale grayish mottling of the retinal pigment epithelium.

The late onset of visual acuity and field loss appeared to be similar to the 2 cases described in the US and Germany. The clinical presentation of the US family with Q344X mutation was described as mild. Three young members in their late 20s were reported to have normal visual acuity and kinetic fields, with no ophthalmoscopic abnormalities. However, subtle electroretinographic abnormalities were recorded with slightly reduced rod but normal cone electroretinogram amplitude, and with 1 log unit of rod sensitivity loss but no cone sensitivity loss across the visual field. Two older family members between 40 and 50 years of age had reduced visual field and acuity, showed retinal attenuation and pigmentary abnormalities in the superior and inferior retina.³² The German patient was also reported to have a mild phenotype, with slight reduction of electroretinography at 5 years old, and clinically RP progressed slowly over the next 13 years with mild alteration in vision acuity, rod sensitivity and visual fields.¹⁹ In contrast to the mild phenotype associated with C-terminal mutation, reports of N-terminal mutations in various populations appeared to share more severe phenotypes.^{33,34} This suggests different functions for the N- or C-terminus of the opsin protein. The similar phenotypic presentation of Q344X mutation in more than one population also suggests the lack of other modifying genes or epigenetic factors in causing the phenotype.

Acknowledgements

We thank the families for their contribution of samples. We also acknowledge the financial support from the Defence Medical and Environmental Research Institute, DSO National Laboratories, Singapore, and the National Medical Research Council, Singapore.

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