

A Missense Mutation in *HK1* Leads to Autosomal Dominant Retinitis Pigmentosa

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PURPOSE. Retinitis pigmentosa (RP) is a genetically heterogeneous disease with over 60 causative genes known to date. Nevertheless, approximately 40% of RP cases remain genetically unsolved, suggesting that many novel disease-causing genes are yet to be identified. In this study, we aimed to identify the causative mutation for a large autosomal dominant RP (adRP) family with negative results from known retinal disease gene screening.

METHODS. Linkage analysis followed by whole-exome sequencing was performed. Stringent variant filtering and prioritization was carried out to identify the causative mutation.

RESULTS. Linkage analysis identified a minimal disease region of 8 Mb on chromosome 10 with a peak parametric logarithm (base 10) of odds (LOD) score of 3.500. Further whole-exome sequencing identified a heterozygous missense mutation (NM_000188.2:c.2539G>A, p.E847K) in hexokinase 1 (*HK1*) that segregated with the disease phenotype in the family. Biochemical assays showed that the E847K mutation does not affect hexokinase enzymatic activity or the protein stability, suggesting that the mutation may impact other uncharacterized function or result in a gain of function of *HK1*.

CONCLUSIONS. Here, we identified *HK1* as a novel causative gene for adRP. This is the first report that associates the glucose metabolic pathway with human retinal degenerative disease, suggesting a potential new disease mechanism.

Keywords: *HK1*, hexokinase, autosomal dominant retinitis pigmentosa (adRP), novel disease-causing gene, next-generation sequencing (NGS)

Retinitis pigmentosa (RP) is an inherited retinal degenerative disease affecting 1 in 4000 people around the world.¹ The typical early symptoms of RP include night blindness and tunnel vision due to the loss of peripheral rod photoreceptor cells. As the disease progresses, cone photoreceptor cells start to degenerate, which may eventually lead to complete blindness.

The genetic basis of RP is highly heterogeneous. To date, mutations in more than 60 genes are known to cause RP.² These genes function in strikingly diverse biological pathways, including phototransduction, transcription regulation, pre-mRNA splicing, and photoreceptor structure maintenance. Various inheritance patterns have also been observed, including autosomal dominant (ad), autosomal recessive (ar), x-linked (xl), digenic, and even mitochondrial modes.³⁻⁶ Despite the

implementation of current next-generation sequencing technologies, approximately 40% of all adRP cases still cannot be explained by mutations in known genes, suggesting that novel disease-causing genes likely exist and remain to be identified.⁷⁻¹¹ In this study, we aimed to identify novel RP disease-causing genes by studying a large adRP family with negative results from known retinal disease gene screening using linkage mapping followed by whole-exome sequencing.

MATERIALS AND METHODS

Clinical Diagnosis of RP

Approval from the Institutional Review Board of the University of California-San Diego was obtained for this study, and

informed consent was obtained from all patients. Twenty-five individuals from one big family participated in the study, and blood samples were obtained. A visual history was obtained from all patients, and best-corrected visual acuities were assessed. Ophthalmoscopic examination was performed on all patients, and fluorescein angiography was done on three affected patients. This study adhered to the Declaration of Helsinki.

Linkage Analysis

Genome-wide linkage analysis was performed using 5913 single nucleotide polymorphism (SNP) sites (Illumina Linkage-24 panel; Illumina, San Diego, CA, USA). PLINK was used to filter SNP sites of either low genotyping rate or Mendelian errors.¹² Linkage analysis was then performed by Merlin using a rare dominant model with 90% penetrance.¹³

Library Preparation and Whole-Exome Sequencing

The Illumina pair-end DNA library preparation followed the standard manufacturer's instructions. Briefly, DNA was quantified using NanoDrop (Thermo Scientific, Wilmington, DE, USA). Total DNA (1 µg) was sheared into fragments of 300 to 500 bp, end-repaired, and had a 3' adenosine base added. Then Illumina Yshaped adapters were added to the DNA fragments, and 10 cycles of PCR reactions were used to amplify the libraries. The library DNA was further quantified using a PicoGreen assay (Life Technologies, Carlsbad, CA, USA). Six library DNA samples were pooled together before the capture step. In total, 3 µg pooled DNA was enriched by the NimbleGen SeqCap EZ Hybridization and Wash kit (NimbleGen SeqCap EZ Human Exome Library v.2.0; NimbleGen, Madison, WI, USA) following the manufacturer's protocols. After that, the postcapture libraries were quantified using PicoGreen assay and then sequenced on an Illumina HiSeq2000 machine.

Bioinformatics Analysis

Data were processed using an in-house bioinformatics pipeline as previously described.^{10,11,14} In particular, raw variants were called using Atlas2 Suite.¹⁵ Considering a dominant model, variants with an occurrence of one or more in any of the variant databases queried, including 1000 Genome,¹⁶ dbSNP135,¹⁷ NHLBI Exome Sequencing database,¹⁸ NIEHS Exome Sequencing database,¹⁹ and an internal control database of 11,000 exomes, were filtered out. dbNSFP v2.3 was used to functionally predict the effects of missense variants.^{20,21}

Sanger Validation

For each identified mutation, a 500-bp flanking sequence at both sides was obtained from the UCSC genome browser (hg19 assembly; <https://genome.ucsc.edu/> [in the public domain]). RepeatMasker was used to mask the repetitive sequence in human genome.²² Primer 3 was used to design a pair of primers for generating a 400- to 600-bp PCR product to sequence the mutation site and at least 50 bp surrounding it.²³ After PCR amplification, the amplicons were sequenced on an ABI 3730xl or 3500xl Genetic Analyzer (Life Technologies).

Biochemical Assays

Hexokinase 1 cDNA was cloned, and the site mutation was generated by Quikchange II XL Site Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The expression and purification followed the protocol described in the

manual. Hexokinase activities were measured using HK colorimetric assay kit (BioVision, Milpitas, CA, USA) with 0.1 µg purified *HK1* proteins. Diagram of the crystal structure of recombinant human hexokinase type I with 1,5-anhydroglucitol 6-phosphate was obtained from the National Center for Biotechnology Information (NCBI) protein database (URL [in the public domain]) with PDB ID 4FPB and analyzed by Cn3D4.3.1, also downloaded from NCBI (<http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?uid=111440>). HEK-293T cell line was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. DNA transfection was performed using lipofectamine 2000 from Invitrogen (Invitrogen, Life Technologies, Carlsbad, CA, USA) and following the manual instructions. Total RNA was isolated by RNeasy Mini Kit (Qiagen, Venlo, Limburg, Netherlands), and mRNA was reverse transcribed to cDNA (SuperScript II kit; Invitrogen). This cDNA was then used for real-time PCR with specific primers complementary to *HK1* (forward primer: 5'-CACCTGTGAGGTTGGACTCA-3'; reverse primer: 5'-CCACCATCTCCACGTTCTTC-3'). Human beta-actin (primers 5'-GTACTTGCCTCAGGAGGAG-3' and 5'-ACTCTTCCAGCCTTCCTTC-3') was simultaneously processed in the same sample as an internal control. A StepOne quantitative PCR (qPCR) machine (Life Technologies) and SYBR Green qPCR kit (Invitrogen) was used for real-time PCR reaction according to the manufacturer's procedure. Cell lysates with 20 µg total proteins were resolved by SDS-PAGE and then blotted with specific antibody for human *HK1* (Cell Signaling Technology, Danvers, MA, USA) at a 1:1000 dilution. Student's *t*-test was used in all statistical analyses, and statistical significance was accepted for *P* values less than 0.05.

RESULTS

A Large adRP Family With Negative Results From Known Retinal Disease Gene Screening Was Recruited

The Caucasian family is from the Midwestern United States with northern European ancestry. Out of the 44 members, 18 are affected. DNA samples were obtained from 25 members, including 11 affected individuals. Among the patients, the onset of reduction in night and peripheral vision ranged from age 5 to 23 (Supplementary Table S1). The disease severity ranged from mild pigmentary changes to severe retinal pigment epithelium and retinal degeneration. In addition, there was concomitant cone photoreceptor degeneration in older patients manifested by photophobia, color vision changes, and decreased central vision. Fundus images of the proband showed an abrupt border of chorioretinal degeneration outside the posterior pole, sclerosis of choroidal vessels, and pigmentary changes in the macula (Fig. 1b).

Using the retinal disease gene sequencing panel developed in our group,²⁴ mutation screening in all known retinal disease-associated genes, including 23 adRP genes known to date, was performed for one proband. Despite good coverage (~30×), no pathogenic mutations in known retinal disease genes were identified for the patient, suggesting that the potential cause may reside in a novel gene (data not shown).

Genome-Wide Linkage Analysis Revealed a Minimal Disease Haplotype Region of 8 Mb on Chromosome 10 With Peak LOD Score of 3.500

In order to narrow down the causative loci, we performed genome-wide linkage analysis using 5913 SNP sites on 14

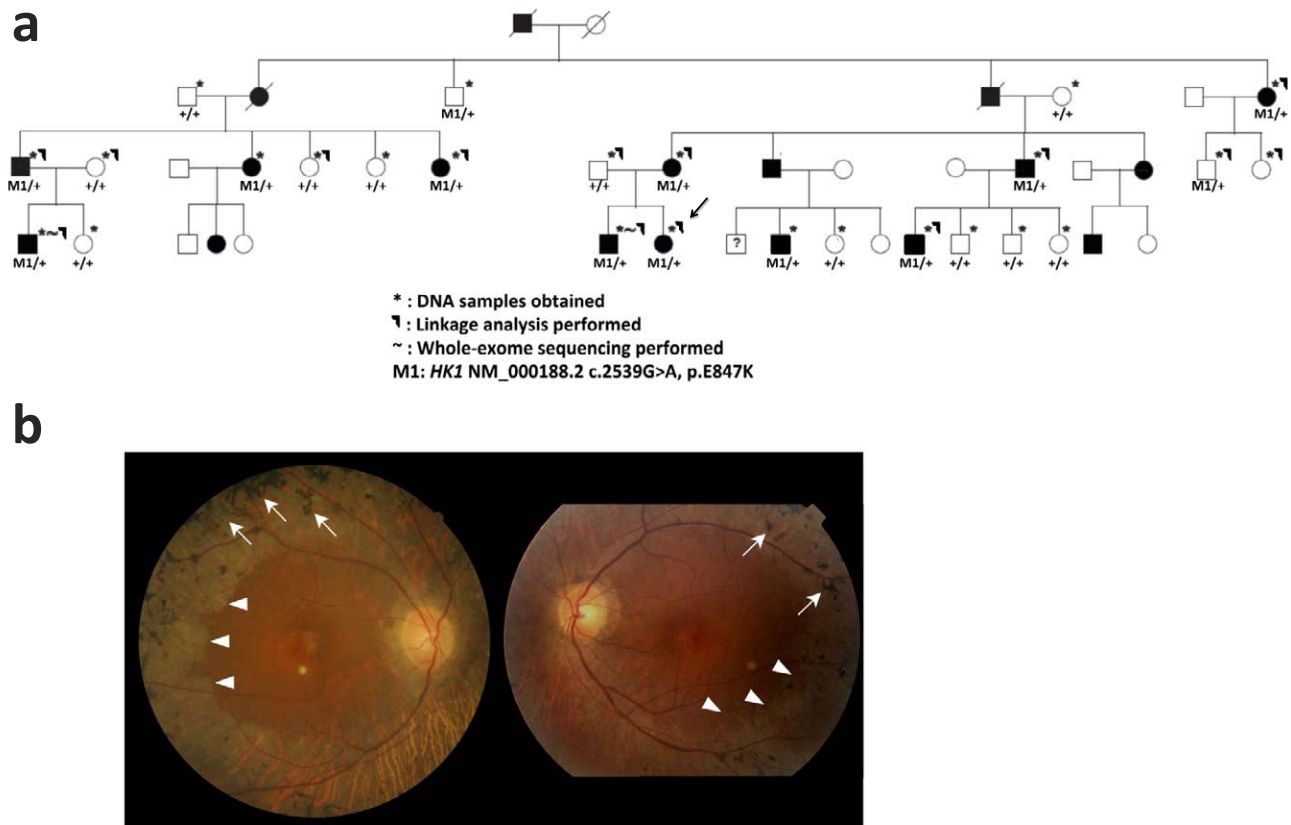


FIGURE 1. (a) Pedigree of the large adRP family. DNA samples were obtained from 25 family members (*). Linkage analysis was performed on 14 family members (¶). Whole-exome sequencing was performed on two affected members (~). The *HK1* E847K mutation segregated with the disease phenotype across the entire family with 85% penetrance. (b) Fundus photos of adRP proband (age 59, decimal visual acuity 0.1 in both eyes, marked by *arrow* in [a]) demonstrate typical bone spicule pigmentation (*arrows*) and extensive retina and RPE atrophy (*arrowheads*). An abrupt border of chorioretinal degeneration outside the posterior pole, sclerosis of choroidal vessels, and pigmentary changes in the macula region were observed. The *pale circles* near the center of the fundus are camera artifacts.

family members, nine of whom were affected (Fig. 1a). Using PLINK,¹² 125 SNP sites were removed from further analysis due to either low genotyping rate or Mendelian errors. The remaining 5788 sites were then analyzed by Merlin using a rare dominant model with 90% penetrance.¹³ A peak parametric logarithm (base 10) of odds (LOD) score of 2.974 was observed on chromosome 10, with a minimal linkage region (LOD score > 0) of 10.4 Mb ranging from marker rs1925622 (chr10: 68769638) to marker rs158421 (chr10: 79182034). To further confirm the linkage mapping and reconstruct the disease haplotype, short tandem repeat (STR) typing was performed across all 25 samples. The combined genotyping boosted the peak parametric LOD score to 3.500 with a minimal disease haplotype region of 8 Mb (Figs. 2a, 2b; Supplementary Fig. S1). It is worth noting that the minimal disease region does not include any known adRP genes. This further excluded the possibility that the causative mutations reside in the known genes.

Whole-Exome Sequencing Identified Missense Allele E847K in *HK1* as the Top Candidate Causative Mutation

To identify the causative mutation, we performed whole-exome capture sequencing of two affected family members (Fig. 1a). High-quality sequencing results were obtained for both samples with a mean coverage of 90× and 83×, respectively. An in-house bioinformatics pipeline was used to

process the raw reads and perform variant calling, filtering, and annotation as described in Material and Methods. A total of only four rare coding-change variants were shared by the two affected members. Surprisingly, three of them were within the linkage region. Among the three candidates within the linkage region, one was a heterozygous missense variant (NM_000188.2: c.2539G>A, p.E847K) in *HK1*. The two other variants (NM_030625: c.598A>G, p.K200E and c.5996A>C, p.H1999P) were in *TET1*. The only shared rare coding-change variant outside the linkage region was a missense change (NM_032292: c.1565T>C, p.M522T) in *GON4L*. All four variants were completely absent in control databases totaling approximately 18,500 individuals (1000 Genomes, ESP6500, and our internal exome sequencing database of 11,000 individuals).

To further prioritize the candidate list, information such as gene expression and variant pathogenicity prediction was utilized. As showed in Supplementary Table S2, *HK1* had significantly higher mRNA expression in the human retina than *TET1* and *GON4L*.²⁵ Meanwhile, in a proteomic study of bovine retina, *HK1* protein was detected in the outer segment of photoreceptor cells, as well as multiple subcellular compartments of the retina, while *TET1* and *GON4L* protein was not detected at all.²⁶ In addition, the *in silico* prediction tool, dbNSFP version 2.3,^{20,21} predicted that the *HK1* variant was likely deleterious to the protein's function, while *TET1* and *GON4L* variants were predicted to be benign (Supplementary Table S3).

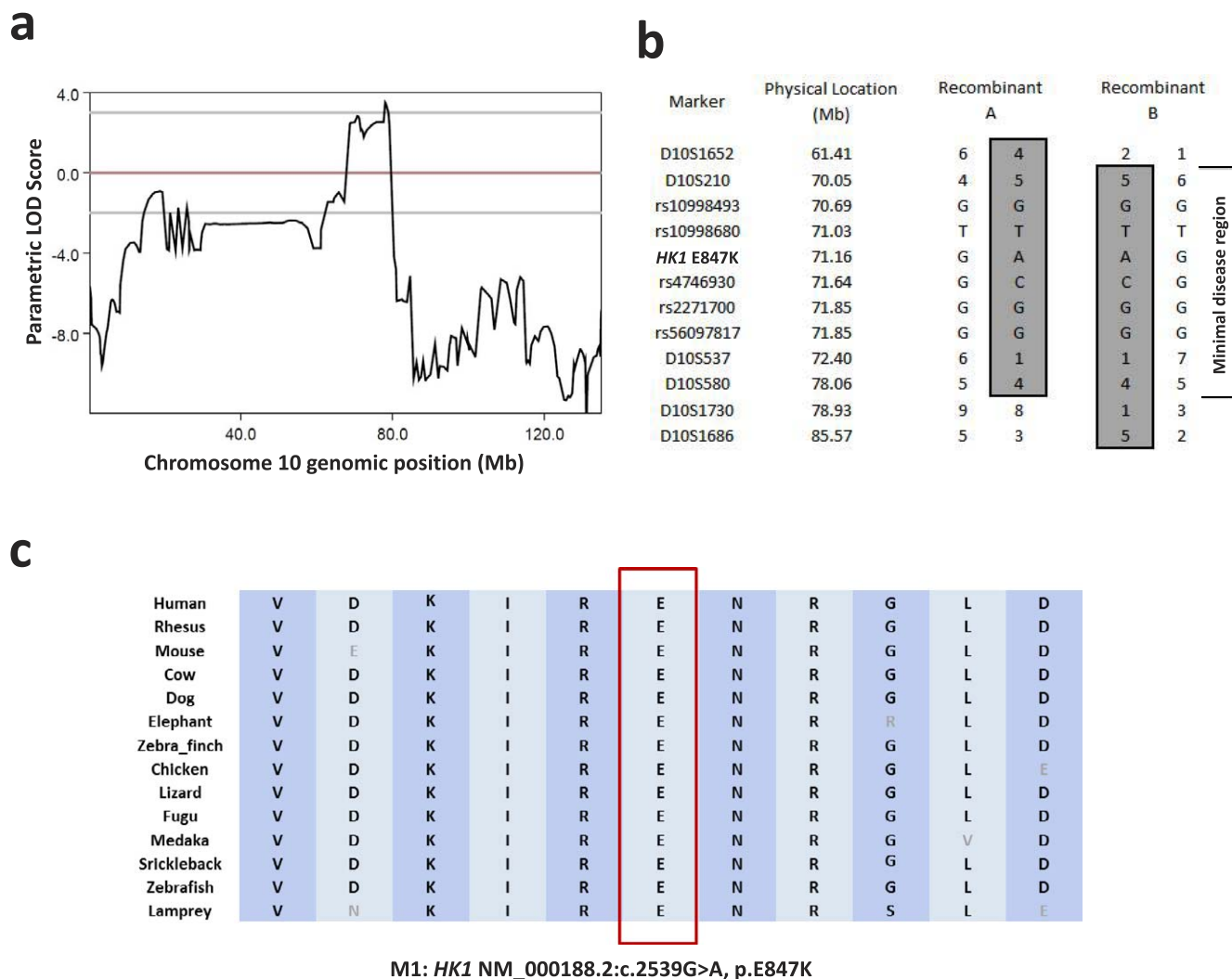


FIGURE 2. (a) A peak parametric LOD score of 3.500 was observed on chromosome 10. (b) A minimal disease haplotype region of 8 Mb was obtained. (c) The *HK1* E847 is highly conserved across vertebrates.

As a result, the *HK1* variant was considered the top candidate causative mutation. Further segregation tests by Sanger sequencing indicated that the *HK1* mutation segregated with the disease phenotype across the entire family with 85% penetrance (Fig. 1a). Two asymptomatic family members, who had the disease haplotype and the *HK1* E847K mutation but did not exhibit the phenotype due to incomplete penetrance, were examined at ages 78 and 37. Their vision was unaffected, and fundus examination did not reveal bone spicule pigmentations. The *HK1* mutation affects an amino acid site that is highly conserved across vertebrates (Fig. 2c). Moreover, the mutation resides in a common exon that is shared by all known *HK1* isoforms including the retinal-specific one; and indeed, RNA-seq analysis indicated that the mutation site was highly expressed in the human retina.²⁵

Biochemical Assays Showed That *HK1* E847K Mutation Did Not Affect Hexokinase Enzymatic Activity or the Protein Stability

To investigate the functional consequences of the *HK1* E847K mutation identified in the adRP family, a series of biochemical assays were performed to test if the mutation would affect

hexokinase enzymatic activity or the protein stability. Wild-type (WT) and mutant *HK1* flag-tag fusion proteins were overexpressed in 293T cells and purified using anti-flag-M2 magnetic beads (Sigma-Aldrich, St. Louis, MO, USA) (Supplementary Fig. S2a). The kinase activities of recombinant *HK1* proteins were then tested using hexokinase colorimetric assay kit (BioVision). The *HK1* mutant protein showed similar hexokinase activity compared to WT protein (Supplementary Fig. S2b). This is consistent with the three-dimensional crystal structure (PDB ID 4FPB) analysis indicating that the mutation site was not located in any kinase activity-associated domain (Supplementary Fig. S2c). In addition to enzymatic activity, mRNA and protein levels of the *HK1* mutant were also measured in 293T cells 2 days after transfection, using RT-qPCR and Western blot analysis. Similar mRNA and protein levels were observed for both mutant and WT *HK1* (Supplementary Figs. S2d, S2e).

DISCUSSION

In this study, we identified for the first time *HK1* as a novel disease-causing gene for adRP, implying the importance of glucose metabolic pathway in human retinal degenerative

diseases. One large multigeneration family was found to carry mutation in *HK1* after linkage analysis followed by whole-exome sequencing. The identified E847K mutation is extremely rare, affects highly conserved amino acid, and was predicted to be detrimental.

Hexokinase 1 encodes one of the four human hexokinases that play essential roles in glucose metabolism. Among them, *HK1* is the most ubiquitously expressed hexokinase gene and is one of the only two that were found to be highly expressed in the human retina. Within the retina, the *HK1* protein is highly enriched in the photoreceptor inner segment, outer plexiform layer, inner nuclear layer, inner plexiform layer, and ganglion cell layer.²⁷ The high abundance and broad distribution of *HK1* suggest its crucial function in satisfying the energy requirement resulting from the high rate of metabolic activity in the retina.

Because of *HK1*'s essential function in glycolysis, complete loss of function (LOF) of *HK1* is hypothesized to be embryonic lethal. Supporting this hypothesis is the fact that only partial LOF mutations in *HK1* have been observed in rare autosomal recessive nonspherocytic hemolytic anemia cases. The pathogenesis is primarily due to the hexokinase enzymatic deficiency in red blood cells.²⁸⁻³⁰ On the other hand, *HK1* has also been implicated in the autosomal recessive Russe type of hereditary motor and sensory neuropathy (HMSNR).³¹ However, in HMSNR patients, the change of hexokinase enzymatic activity was not evident, leaving the possibility that the causality involves a novel function of *HK1*.³¹

In our study, biochemical assays showed no obvious difference between the E847K mutant and WT allele in terms of both enzymatic activity and mRNA and protein expression level, suggesting that hexokinase deficiency was not likely to be the underlying mechanism. Indeed, our patients with the E847K mutation did not show any sign of anemia. In addition, no signs of exercise intolerance or brain cognition deficits were observed. Taken together, given the dominant inheritance in nature, we propose that the mutation identified in our study is likely to lead to defect in *HK1*'s retina-specific function or result in a gain of function.

Several studies of hexokinase already suggest possible hypotheses for *HK1*'s molecular pathogenesis in retinal degeneration. Studies in the mouse retina show that mitochondrial metallochaperone *Cox11*, chaperone protein *Ranbp2*, and *Hk1* interact with each other in equilibrium to maintain metabolic activity and normal retina function.³² It is possible that mutations in *HK1* could abolish such interaction, causing disturbance of the equilibrium and resulting in retinal degeneration. Another hypothesis comes from a recent study showing that light activation of insulin receptors can regulate the hexokinase-mitochondria interaction and inhibit apoptosis of photoreceptors.³³ It is possible that mutations in *HK1* could disrupt the regulation from insulin receptors, causing dissociation between hexokinase and mitochondria that would eventually trigger photoreceptor degeneration. If that is the case, enhancing the hexokinase-mitochondria association may be a potential therapeutic approach.

In summary, we report, for the first time, *HK1* as a novel disease-causing gene for adRP. The identified missense mutation causes a glutamate-to-lysine change at position 847. Surprisingly, an identical mutation (E847K) in *HK1* was identified as the top candidate for another large adRP family by an independent group, and there is no sign that the two families are related (Daiger SP, written communication, 2014). This further strengthens our conclusion that the E847K mutation in *HK1* is a rare cause for adRP.

Web Resources

PLINK: <http://pngu.mgh.harvard.edu/~purcell/plink/>.

Merlin: <http://www.sph.umich.edu/csg/abecasis/Merlin/index.html>.

RetNet: <http://www.sph.uth.tmc.edu/Retnet>.

1000 Genome: <http://www.1000genomes.org/>.

ESP6500: <http://evs.gs.washington.edu/EVS/>.

dbNSFP: <https://sites.google.com/site/jpopgen/dbNSFP>.

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