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Novel insights into the contributions of isocitrate dehydrogenases to the Krebs cycle from patients with retinitis pigmentosa

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Abstract

We describe two families with retinitis pigmentosa (RP), a hereditary neurodegeneration of rod and cone photoreceptors in the retina, in which affected members were homozygotes for loss-of-function mutations in the *IDH3B* gene encoding the beta subunit of NAD-specific isocitrate dehydrogenase (NAD-IDH), the enzyme believed to catalyze the oxidation of isocitrate to α -ketoglutarate in the citric acid cycle. Cells from the affected family members had a substantial reduction of NAD-IDH activity with about a 300-fold increase in the K_m for NAD. NADP-specific isocitrate dehydrogenase (NADP-IDH), an enzyme that can catalyze the same reaction, was normal. The patients had no health problems associated with the enzyme deficiency except for RP. The existence of these patients supports the hypothesis that mitochondrial NADP-specific IDH, rather than NAD-IDH, can serve as the major catalyst for this reaction in the citric acid cycle outside the retina, and that the retina has a particular requirement for NAD-IDH.

Mutations in at least 34 genes have been identified as causes of nonsyndromic RP, including 15 dominant genes, 20 recessive genes, and 2 X-linked genes (RetNet website). The identified RP genes are estimated to account for about 60% of cases¹. In addition, 11 unidentified genes have been mapped to specific chromosomal regions. It is estimated that there are dozens of still unmapped, unidentified recessive RP genes, each accounting for at most a few percent of cases^{1, 2}.

The approach we used to search for some of the unidentified recessive RP genes depends on the fact that recessive alleles are often deletions, frameshift mutations, or nonsense mutations that result in a scarcity of the disease gene's transcript either because the transcript is not made

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AUTHOR CONTRIBUTIONS D.T.H., T.L.M., and T.P.D. designed and conducted the molecular genetic analyses. E.L.B. clinically evaluated, selected, and recruited patients and their family members, as well as helped in the design of the study. M.D. and R.F.C. designed and conducted the enzyme assays. All authors discussed and interpreted the results and wrote the manuscript.

URLs. RetNet, <http://www.sph.uth.tmc.edu/retnet/>; EyeSAGE, <http://neibank.nei.nih.gov/EyeSAGE/>; *IDH3B* splicing variants, http://genome.ucsc.edu/cgi-bin/hgTracks?position=chr20:2587041-2592843&hgid=107503458&refGene=pack&hgFind.matches=NM_006899; Primer3, http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi

or is rapidly degraded due to nonsense-mediated decay³. The principle underlying this method has been previously described^{4, 5, 6}, but, as far as we know, the method has never been used to identify a novel gene causing a hereditary disease. Using microarray techniques that simultaneously assay mRNA levels from tens of thousands of transcripts in individual patients, we searched for genes with absent or very low expression that may be due to two allelic disease-causing mutations. While it would have been ideal to carry out the analysis with RNA derived from the retina prior to its degeneration, this tissue is not available from patients. However, a substantial minority of the already identified RP-causing genes, such as *RPGR*⁷, *RP2*⁸, *IMPDH1*⁹, *PRPF31*¹⁰, *PRPF8*¹¹, and *PRPF3*¹² are expressed throughout the body, so we reasoned that low-abundance messages from some yet-to-be-identified RP genes might be detected by analyzing lymphoblast mRNA.

We focused our study on 13 unrelated recessive RP families that were previously screened with negative results for mutations in all or all but one of the following known recessive RP genes: *PDE6A* (accounting for approximately 3-4% of recessive RP cases), *PDE6B* (4-5%), *RPE65* (2%), *TULP1* (1%), *USH2A* (8%), and *CNGA1* (1%)¹. Six of the 13 families had 2 or more available affected siblings: one sibship had 4 affected members and 5 sibships each had 2 affected members. Such multiplex sibships were preferred for this study because if any of them had recessive mutations leading to low-abundance messages, the low-abundance mRNA would be shared by all affected siblings and thus be more likely to be recognized as distinct from fortuitously low-abundance messages from genes other than the disease gene. Lymphoblast cell lines were created from peripheral blood lymphocytes from the 13 index patients as well as from their available affected siblings and some unaffected relatives. Lymphoblast lines were also prepared from 4 control individuals: 2 were unrelated patients with Usher syndrome who were homozygous for the *USH2A* mutation Glu767SerfsX21 (c.2299delG), one was a patient with dominant RP and the Pro23His (c.68C>A) mutation of the rhodopsin gene, and one was a person without RP or a family history of RP.

Lymphoblast mRNA was labelled and hybridized to the Affymetrix chip “U133Plus 2.0” which has one or more sets of oligomer probes to individually assay over 47,000 human transcripts derived from approximately 39,500 genes or ESTs. Normalized signal intensities measure the relative amount of patient mRNA bound to each probe. Applying stringent filtering criteria to the numerous genes in each lymphoblast line for which the microarray analysis indicated low mRNA levels, we identified 3 to 25 candidate genes or ESTs in each family, for an aggregate of 50 candidates in the multiplex families (Supplementary Table 1). We individually eliminated 13 of the 50 corresponding genes as likely disease genes because we found no mutations by direct sequencing or because our analysis of intragenic polymorphisms showed that alleles did not co-segregate with disease among the relatives of the relevant index patient (Supplementary Table 2). Analysis of the remaining candidate genes has not been completed.

We concurrently evaluated the microarray results from 7 families in which only one affected patient provided a blood sample for a lymphoblast line. The *IDH3B* gene¹³ was the most remarkable gene from those families because the 3 probe sets (210014_x_at, 210418_s_at, and 201509_at) that hybridized to the *IDH3B* transcript in index patient 003-053 gave normalized hybridization signals of 62, 25, and 68 in comparison with the signals in the 24 other samples of 384 ± 44 , 279 ± 37 , and 200 ± 15.4 (mean $\pm$ SD), respectively (Fig. 1). This patient had an affected brother who had previously donated a blood sample for DNA analysis but had since died at age 71 years from carotid artery occlusion; lymphoblast cell lines had not been made. Sequencing *IDH3B* revealed that both the index patient and her deceased, affected brother were homozygotes for a one-base-pair deletion in codon 197 (c.589delA; p.Ile197fs; m.p.Ile163fs [m.p. = mature protein]; cDNA numbering is based on mRNA variant 1 which is the longest of three known transcripts). The frameshift produced a premature stop codon in all three mRNA

variants transcribed from *IDH3B* (Fig. 2). The patient's parents were first cousins. Four unaffected siblings were heterozygotes and one was wild-type (Fig. 3).

We subsequently sequenced all 12 exons of *IDH3B* in 261 recessive and 285 simplex RP patients (most simplex cases have autosomal recessive disease). One patient (003-178) was found to be a homozygote for the missense mutation m.p.Leu98Pro (c.395T>C; p.Leu132Pro) in exon 5 (Fig. 2). This patient's parents were first cousins. The patient's three siblings, all unaffected, were heterozygotes (Fig. 3). Neither the m.p.Leu98Pro mutation nor the m.p.Ile163fs mutation was found upon screening the leukocyte DNA from 95 control individuals without RP.

Patients 003-053 and 003-178 were ophthalmologically examined at ages 47 and 38 years, respectively. Both patients had subnormal visual acuities, concentrically constricted visual fields, fundi typical of RP (pale optic discs, attenuated arterioles, and intraretinal pigment deposits), impaired dark-adaptation, and reduced ERG amplitudes indicating substantial loss of rod and cone photoreceptor function. (See the **Supplementary Note** for additional clinical details.) The patients reported no other relevant health problems, and in particular, no problems typically associated with mitochondrial dysfunction (reduced muscle strength, cardiac dysrhythmias, reduced athletic stamina, etc.)

IDH3 catalyzes the oxidative decarboxylation of isocitrate to produce α -ketoglutarate while converting nicotinate adenine dinucleotide (NAD⁺) to NADH in the citric acid cycle, also referred to as the Krebs cycle. IDH3 is a heterotetramer with 2 alpha subunits, 1 beta subunit, and 1 gamma subunit¹⁴. In patient 003-053, with the m.p.Ile163fs mutation, the beta subunit is presumably poorly expressed (as indicated by the low message levels detected by the microarray analysis). Any *IDH3B* protein that would be produced would be truncated and unlikely to participate in a functional heterotetramer. To confirm this prediction and also to determine the effect of the missense mutation m.p.Leu98Pro, we evaluated NAD-IDH activity in lymphoblast cell lysates from the index patients as well as from some of their heterozygote siblings and from normal individuals. The NAD-IDH activities of the homozygotes under standard assay conditions were only 4.8% (m.p.Ile163fs) and 2.4% (m.p.Leu98Pro) of the normal controls, whereas the heterozygotes had respective activities 24% and 82% of normal (Fig. 4). The K_m for NAD in the homozygotes was 267-298 times normal, while it was 4-8 times normal in the heterozygotes (Fig. 4). The concentration of NAD in mammalian tissues has been estimated at 0.4-0.8 mM¹⁵, which is sufficient for normal NAD-IDH with its K_m for NAD around 40 μ M, but is too low to appreciably bind to the mutant NAD-IDH of the homozygote patients which we found to have K_m values of about 11-12 mM.

As shown in Fig. 4, without ADP, the K_m for isocitrate was 3-4 times normal in the homozygotes, while it was normal in heterozygotes. ADP is an allosteric activator for IDH3, which lowers its K_m for isocitrate¹⁶. In our assays, it decreased the K_m for isocitrate by 9-fold in normal controls but only decreased it by 3-fold in the m.p.Ile163fs homozygotes and 5-fold in the heterozygotes. This allosteric effect was completely lost in the homozygote m.p.Leu98Pro mutant cells (Fig. 4). The importance of the region around residue m.p.Leu98Pro for ADP's activation of IDH is supported by previous studies of an engineered substitution of the neighboring residue m.p.Arg99 (p.Arg133). The mutant m.p.Arg99Gln, like the m.p.Leu98Pro mutant, lacked an allosteric effect of ADP¹⁷.

In the archetypical model of the citric acid cycle, NAD-IDH (IDH3) is the enzyme catalyzing the conversion of isocitrate to α -ketoglutarate, an essential reaction of the cycle that simultaneously changes a molecule of NAD⁺ to NADH¹⁸. The NADH produced in this step and other steps of the citric acid cycle is used to generate ATP, a molecule universally used in cells as an energy source. It is therefore noteworthy that our patients had no evident health

problems except for RP. Another mitochondrial enzyme, nicotinamide adenine dinucleotide phosphate (NADP)-IDH (IDH2), also converts isocitrate to α -ketoglutarate but uses the cofactor NADP⁺ instead of NAD⁺. NADP-IDH activity and K_ms were normal in our patients (Supplementary Table 3). The NADPH produced by NADP-IDH can be converted into NADH through the action of nicotinamide-nucleotide transhydrogenase (NNT) which is present in the inner membrane of mitochondria^{19, 20}. The activity of NADP-IDH exceeds that of NAD-IDH in mammalian mitochondria²⁰, and the activity of the transhydrogenase is comparable to the rate of NADPH production by NADP-IDH. It is possible that in our patients, NADP-IDH together with the transhydrogenase adequately substitute for the defective function of NAD-IDH in all tissues except the retina. In fact, our review of SAGE tags (EyeSAGE), which crudely reflect mRNA expression levels, indicates that NADP-IDH (IDH2) tags are more frequent than NAD-IDH (IDH3) tags across all human tissues except the retina and retinal pigment epithelium (Fig. 5).

An alternative explanation for the viability of humans with greatly reduced NAD-IDH is that NADP-IDH may actually be the principal catalyst of the isocitrate to α -ketoglutarate reaction in the citric acid cycle in all tissues, and IDH3 serves as an accessory enzyme that augments or regulates the reaction, with the retina being particularly sensitive to the loss of this accessory activity. In this regard, it is notable that the K_m for isocitrate is about 1 μ M for NADP-IDH which is low relative to the 40-60 μ M of isocitrate estimated to be present in mammalian mitochondria²¹. Thus, NADP-IDH would normally be saturated with isocitrate. Furthermore the K_m for NADP is $\sim$ 2 μ M, while the mammalian tissue levels of NADP are estimated at $\sim$ 100 μ M¹⁵, so the coenzyme site will also be saturated. In contrast, for NAD-IDH the K_m for isocitrate is 1-2 mM (in the absence of ADP), which is high relative to the mitochondrial levels of isocitrate; it is only in the presence of ADP that the K_m for isocitrate is lowered into the 0.1-mM range, thereby allowing that enzyme to make a contribution to the oxidative decarboxylation of isocitrate. These considerations all support the interpretation that NADP-IDH can be the major enzyme serving this step of the citric acid cycle, with the retina being a possible exception. The association of loss-of-function mutations with RP suggests that NAD-IDH is essential for normal retinal function. Further studies of mitochondrial metabolism in the retina and other tissues should help to confirm the association of IDH3 mutations with RP and the role of the IDH family of enzymes in mitochondrial metabolism.

METHODS

Patients

Twenty-one recessive RP patients from 13 unrelated families and 4 controls were included in the study. This study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Boards of the Massachusetts Eye and Ear Infirmary and Harvard Medical School, and the Human Subjects Review Board of the University of Delaware. Informed consent was obtained from all study participants. See the **Supplementary Note** for further details regarding patient recruitment and phenotyping criteria.

Lymphoblast cell lines were created from whole blood by immortalization with Epstein-Barr virus. Cells were grown to confluence, pelleted, suspended in Trizol (Invitrogen, Carlsbad, CA) and stored at -80°C until RNA was extracted.

Methods for RNA isolation, labeling, and hybridization other than indicated in the text are provided in the **supplementary methods**.

Statistical Analysis

Raw data reflecting hybridization intensities, from individual probes of 25 different chips were provided as Microarray Suite (MAS) 5.0 experiment data files (.CHP, .TXT, .RPT, .EXP, .CEL), and were normalised using d-Chip²² (all arrays are normalized to a chip with median overall brightness). The normalized data were extracted into a Microsoft Excel file and hybridization intensities from all probes of the affected patients in individual families were compared to all of the other samples. Significance in multiplex families was tested using a one-tailed Student's *t*-test assuming unequal variance, a Bonferroni correction and P-value < 0.05. In families with only one available patient, the transcript in that patient had to have a hybridization signal more than two standard deviations below the mean hybridization intensity in all other patients.

DNA sequencing of candidate genes was performed by amplifying individual exons along with flanking intron sequences using primers designed with 'Primer 3'. The amplified fragments were treated with ExoSAP-IT (US Biochemical, Cleveland OH) to eliminate unincorporated primers and dNTPs and were directly sequenced using BigDye version 3.1 and an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA). For some genes, only exons with one or more known single nucleotide polymorphisms (SNPs) were amplified and sequenced in order to determine if the alleles cosegregated with disease in the relevant families.

Protein extraction was performed by resuspending each lymphoblast cell pellet from a 200-ml culture (see **Supplementary methods** for a description of lymphoblast culturing) in 1 ml Mammalian Protein Extraction Reagent (M-PER - Pierce, Rockford, IL). The volume of each cell pellet ranged from approximately 300-400 µl and yielded between 9-15 mg of protein. The cells were incubated on ice during lysis for 1 hour. The lysate was centrifuged and the supernatant was used for measuring enzyme activity and protein concentration. Protein concentration was determined from O.D._{280nm} after correcting for the ratio of O.D._{280/260nm}²³.

Activity of NAD-IDH and NADP-IDH was determined using the Perkin-Elmer fluorescence spectrophotometer MPF-3 by monitoring the time-dependent increase in the fluorescence of NADH or NADPH at 440 nm upon excitation at 340 nm. Standard assay conditions for NAD-IDH were 33 mM Tris-acetate buffer, pH 7.2, 20 mM DL-isocitrate, 1 mM NAD and 1 mM MnSO₄ as final concentrations. Kinetic studies were conducted by varying the concentration of one substrate at a time. Measurements of the Km for isocitrate in the absence and presence of 1 mM ADP were performed at 10 mM NAD. Standard assay conditions for NADP-IDH were 30 mM triethanolamine hydrochloride buffer, pH 7.4, 4 mM DL-isocitrate, 0.1 mM NADP and 2 mM MnSO₄. The Km-isocitrate and Km-NADP were determined by varying the concentration of each substrate at otherwise standard conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Accession numbers. IDH3B (chromosome 20p13) (NCBI) Entrez Nucleotide isoforms: NM_006899 (transcript variant 1), NM_174855 (transcript variant 2), NM_174856 (transcript variant 3); Entrez Gene ID: 3420; OMIM ID: 604526). Gene Expression Omnibus: mRNA expression data have been deposited with accession code GSE12086.

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Figure 1.

Scatter plot of probe-set hybridization intensities from individual 003-053. Data are organized according to percentage of respective mean signal across all subjects (x axis) and number of s.d. below this mean (y axis). The plot shows the 306 probe sets detecting hybridization signals two or more s.d. below the mean of all other subjects and controls. Of the 306 probe sets, 3 were clear outliers, with hybridization intensities more than 6 s.d. below the respective means; the 3 probe sets detected IDH3B (IDH3B-1, probe set 210014_x_at; IDH3B-2, 210418_s_at; IDH3B-3, 201509_at.) These were the only probe sets in the microarray designed to hybridize to mRNA from IDH3B.

Figure 2.

IDH3B showing three known RNA splicing variants (see URLs section below) and the location of the m.p.Ile163fs and m.p.Leu98Pro mutations. The m.p.Leu98Pro mutation is a missense mutation and alters *IDH3B* in variants 1 and 2. There is no predicted effect on variant 3 since the mutation is located in the 5' untranslated region of that variant. Splice variant 3 is missing exons 1-5 which encode 132 amino acid residues out of 385 in the full-length protein. It is not likely to encode a functional beta subunit that could compensate for the defective m.p.Leu98Pro mutation in the two other variants. The m.p.Ile163fs mutation causes a frameshift with a premature stop codon in all three variants presumably leading to mRNA degradation by nonsense-mediated decay.

Figure 3.

Pedigrees with the *IDH3B* m.p.Ile163fs and m.p.Leu98Pro mutations. Black arrows point to the index patients. Beneath each family member's symbol is that person's *IDH3B* genotype; family members for which no genotype is shown did not submit a blood sample for analysis. Red arrows indicate the location of the mutation in the DNA sequence tracings. Nucleotides corresponding to the normal sequence (top line) and mutated sequences (lower line) are indicated below the sequence alignments.

Figure 4.

Activity of NAD-dependent isocitrate dehydrogenase. Average results of at least duplicate measurements with standard deviations of Km for NAD and Km for isocitrate (with or without ADP), Vmax and NAD-IDH activity under standard conditions in lymphoblast lysates from three normal subjects (+/+); two heterozygotes (m1/+) and one homozygote (m1/m1) with *IDH3B*-m.p.Ile163fs; one heterozygote (m2/+) and one homozygote (m2/m2) with the *IDH3B*-m.p.Leu98Pro mutation. The mutant homozygotes have a 267-298-fold increase in the Km for NAD (upper left panel) and a resulting loss of NAD-IDH activity (lower right panel). The normal effect of ADP in lowering the Km for isocitrate is partially (for m1 = m.p.Ile163fs) or completely (for m2 = m.p.Leu98Pro) lost in homozygote patients (lower left panel).

Figure 5.

Frequency of SAGE tags from IDH3 and IDH2 in the retina and retinal pigment epithelium (retina/RPE) in comparison to that from all other human tissues using EyeSAGE database. IDH3: mitochondrial NAD-dependent isocitrate dehydrogenase, alpha subunit - *IDH3A* (gene ID 3491), beta subunit - *IDH3B* (gene ID 3420), gamma subunit - *IDH3G* (gene ID 3421); IDH2: mitochondrial NADP-dependent IDH - *IDH2* (gene ID 3418). The low ratio of tags from NADP-IDH suggest that the retina/RPE has a relatively low abundance of NADP-IDH and may be unusually dependent on NAD-IDH.