Diagnostic application of clinical exome sequencing in Leber congenital amaurosis

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Purpose: Leber congenital amaurosis (LCA) is a hereditary retinal dystrophy with wide genetic heterogeneity. Next-generation sequencing (NGS) targeting multiple genes can be a good option for the diagnosis of LCA, and we tested a clinical exome panel in patients with LCA.

Methods: A total of nine unrelated Korean patients with LCA were sequenced using the Illumina TruSight One panel, which targets 4,813 clinically associated genes, followed by confirmation using Sanger sequencing. Patients' clinical information and familial study results were obtained and used for comprehensive interpretation.

Results: In all nine patients, we identified pathogenic variations in LCA-associated genes: *NMNAT1* (n=3), *GUCY2D* (n=2), *RPGRIP1* (n=2), *CRX* (n=1), and *CEP290* or *SPATA7*. Six patients had one or two mutations in accordance with inheritance patterns, all consistent with clinical phenotypes. Two patients had only one pathogenic mutation in recessive genes (*NMNAT1* and *RPGRIP1*), and the clinical features were specific to disorders associated with those genes. Six patients were solved for genetic causes, and it remains unclear for three patients with the clinical exome panel. With subsequent targeted panel sequencing with 113 genes associated with infantile nystagmus syndrome, a likely pathogenic allele in *CEP290* was detected in one patient. Interestingly, one pathogenic variant (p.Arg237Cys) in *NMNAT1* was present in three patients, and it had a high allele frequency (0.24%) in the general Korean population, suggesting that *NMNAT1* could be a major gene responsible for LCA in Koreans.

Conclusions: We confirmed that a commercial clinical exome panel can be effectively used in the diagnosis of LCA. Careful interpretation and clinical correlation could promote the successful implementation of clinical exome panels in routine diagnoses of retinal dystrophies, including LCA.

Leber congenital amaurosis (LCA; OMIM 204000) is an inherited ophthalmologic disorder characterized by severe visual impairment in early infancy, nystagmus, absent or sluggish pupillary responses, high hyperopia, and extinguished electroretinogram (ERG) [1,2]. LCA is estimated to affect 1 in every 50,000 to 80,000 individuals and accounts for 20% of blindness in school-age children [3]. To date, mutations in 24 genes have been reported to cause LCA, and more causative genes continue to be identified (assessed September 2016, RetNet) [2,4-7]. These genes encode proteins involved in the processes and pathways associated with phototransduction, retinoid cycle, photoreceptor morphogenesis, guanine synthesis, signal transduction, outer segment phagocytosis, coenzyme NAD synthesis, and intraphotoreceptor ciliary transport.

Genetic testing can definitively diagnose LCA and could provide a basis for future gene therapy. Sequencing analyses of the known causative genes could identify approximately 70% of LCA cases [2,8]. However, the genetic heterogeneity represented by the large number of associated genes leads to difficulties in molecular diagnosis. Moreover, LCA should be differentiated from several other systemic disorders, such as Joubert syndrome, Zellweger syndrome, neuronal ceroid lipofuscinosis, and Senior-Loken syndrome, which exhibit similar ocular phenotypes in early infancy [9,10]. Next-generation sequencing (NGS) technologies have enabled the simultaneous detection of multiple candidate genes, and several laboratories provide gene panel sequencing or whole-exome sequencing (WES) [11].

Disease-specific gene panel sequencing can be designed to sequence almost all coding regions of the genes that cause LCA with high coverage and reliability. However, it cannot detect gene mutations for disorders with overlapping phenotypes for differential diagnosis or detect mutations in other unidentified genes. Such shortcomings can be overcome by WES; however, using WES in diagnostic testing is

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challenging because of the high burden of interpreting a large number of variants of uncertain significance (VUS), issues with reporting incidental findings, reduced coverage and reliability in some regions of disease-specific genes, increased cost, and prolonged time to obtain final results [12].

Sequencing a few thousand genes with known clinical implications, often called clinical exome sequencing, could reduce the analytical burden of WES. In this report, we evaluate the clinical utility of a commercial clinical exome panel in patients with LCA.

METHODS

Patients and families: A total of nine unrelated children with LCA were recruited at Severance Hospital from June 2015 to January 2016. The research protocol was approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine. This study adhered to the tenets of the Declaration of Helsinki. All patients were diagnosed with LCA using the following criteria: 1) early onset severe visual impairment during the first year of life, 2) amaurotic pupil accompanied by nystagmus or wandering eye movement, 3) extinguished or severely reduced ERG, and 4) exclusion of other systemic diseases [1]. ERG examination was performed with an oral sedative, chloral hydrate, if patients were uncooperative. ERG protocols adhered to International Society for Clinical Electrophysiology of Vision standards [13], with some modifications for young infants. All patients were offspring of asymptomatic Korean parents, and familial genetic testing of the parents was performed in seven families.

Library construction and targeted sequencing: Briefly, peripheral blood was collected from basilic or cephalic vein in antecubital fossa, with the use of extension tube if needed. Whole blood was stored at room temperature for less than 2 days before it was proceeded to DNA extraction. Genomic DNA was extracted from leukocytes of whole blood samples using the QIAamp Blood DNA mini kit (Qiagen, Venlo, the Netherlands) according to the manufacturer's instructions which used spin-column procedure with automation on the QIAcube instrument (Qiagen). Intact DNA was quantified and adjusted to a concentration of 5 ng/µl using a Qubit 2.0 fluorometer (Invitrogen, Waltham, MA) and the Qubit dsDNA HS assay kit (Invitrogen). Targeted NGS was performed on the DNA using the Illumina TruSight One sequencing panel on the MiSeq platform (Illumina, San Diego, CA). Briefly, precapture libraries were constructed according to the manufacturer's sample preparation protocol. Each patient's genomic DNA was fragmented with a median size of 300 bp. The DNA fragments were end-repaired, phosphorylated, and adenylated on the 3′ ends. The index adaptors were ligated to the repaired ends, DNA fragments were amplified, and fragments of 200 to 500 bp were isolated. Pooled libraries were sequenced on a MiSeq sequencer (Illumina) using the MiSeq Reagent kit v2 (300 cycles).

Gene targets and NGS data analysis: The TruSight One sequencing panel provides targeted sequencing for 4,813 genes associated with known clinical phenotypes and includes 22 causative genes for LCA (*AIPL1* Gene ID 23746, OMIM 604392; *CABP4,* Gene ID 64802,OMIM 608700; *CEP290* Gene ID 80184, OMIM 610142; *CRB1* Gene ID 23418, OMIM 604210; *CRX* Gene ID 1406, OMIM 602225; *GDF6* Gene ID 392255, OMIM 601147; *GUCY2D* Gene ID 3000, OMIM 600179; *IFT140* Gene ID 9742, OMIM 614620; *IQCB1* Gene ID 9657, OMIM 609237; *KCNJ13* Gene ID 3769, OMIM 603208; *LCA5* Gene ID 167691, OMIM 611408; *LRAT* Gene ID 9227, OMIM 604863; *NMNAT1* Gene ID 64802, OMIM 608700; *PRPH2* Gene ID 5961, OMIM 179605; *RD3* Gene ID 343035, OMIM 180040; *RDH12* Gene ID 145226, OMIM 608830; *RPE65* Gene ID 6121, OMIM 180069; *RPGRIP1* Gene ID 57096, OMIM 605446; *SPATA7* Gene ID 55812, OMIM 609868; *TULP1* Gene ID 7287, OMIM 602280; *IMPDH1* Gene ID 3614, OMIM 146690, and *OTX2* Gene ID 5015,OMIM 600037) listed in RetNet (last accessed September 2016). Two recently discovered genes, *DTHD1 (*Gene ID 401124, OMIM 616979*)* and *CLUAP1 (*Gene ID 23059, OMIM 616787*)* [14,15], are not included in the panel.

Data analysis was performed according to the default parameters of the Illumina MiSeq Reporter software. The Burrows-Wheeler Aligner (BWA)-MEM algorithm was used to read and map the raw sequence data, and the Genome Analysis Toolkit (GATK) was used for variant calling. For each subject, a vcf file containing variant calls was generated, reviewed, and filtered. For every equivocal call in the vcf files, a visual inspection of the mapped data was performed using the Integrated Genomics Viewer 2.3 software (IGV; Broad Institute, Cambridge, MA). For an overview of the sequencing coverage, position-wise coverage values were calculated from each bam file using the SAMtools software. Coverage information was calculated and plotted using the R statistics software.

GRCh37 (hg19) was used as the reference sequence for mapping and variant calling. Databases used for analysis and variant annotation include the Online Mendelian Inheritance in Man (OMIM), Human Gene Mutation Database, ClinVar, Single Nucleotide Polymorphism database (dbSNP), 1000 Genome, Exome Aggregation Consortium (ExAC), Exome Sequencing Project, and Korean Reference Genome Database, with a minor allele frequency cut-off of 0.5% [16]. The

Patient	Total aligned reads	Targeted aligned reads	Targeted aligned bases (bp)	Mean coverage	>=Q30 base pair	Median fragment length (bp)
P1	12,248,376	8,305,029	887,342,110	74.3×	91.8%	308
P2	12,514,079	8,734,402	927,395,332	77.6×	92.5%	279
P3	16,477,550	11,991,255	1,235,952,620	103.5×	93.3%	256
P4	14,490,845	10,323,509	1,074,284,233	89.9×	92.9%	267
P5	19,383,130	13,549,987	1,411,822,212	118.2×	88.1%	271
P6	14,609,502	10,076,663	1,039,201,848	87.0×	94.9%	279
P7	13,891,487	9,961,705	1,026,703,521	85.9×	90.0%	259
P8	13,675,583	10,242,904	1,022,195,973	85.6×	90.9%	225
P9	17,416,972	13,005,976	1,312,819,546	109.9×	93.6%	233
Average	14,967,503	10,687,937	1,104,190,822	92.4×	92.0%	264

TABLE 1. QUALITY CONTROL METRICS OF NEXT GENERATION SEQUENCING RUNS.

Abbreviation: bp, base-pair

pathogenicity of missense variants was predicted using the following in silico prediction algorithms: Sorting Tolerant from Intolerant (SIFT), Polymorphism Phenotyping v2 (PolyPhen-2), and Mutation Taster.

Validation with Sanger sequencing: All pathogenic variations or VUS with high priority were validated with Sanger sequencing, for the proband and parent samples.

RESULTS

Pathogenic variants identified with NGS analysis using TruSight One panel: Quality metrics of the NGS runs performed in the nine patients are summarized in Table 1. More than 14 million reads were sequenced per sample, and approximately 10 million reads were mapped on the target regions. Horizontal coverage, the percentage of regions with more than 20X coverage, was 92.8%.

In six of the nine patients, pathogenic variants in LCA-associated genes were detected in accordance with inheritance patterns. In the remaining three patients, only a single pathogenic variant for each gene was identified (Table 2). P1 had a single pathogenic variant in *CRX*, and a trio study revealed a de novo occurrence. Five patients were compound heterozygous for recessive genes: *GUCY2D* (P2 and P3), *NMNAT1* (P4 and P5), and *RPGRIP1* (P9). For patients with available parental samples (P2, P3, P4, and P5), all compound heterozygous mutations were confirmed to be inherited each from the carrier mother and the carrier father.

In P7, two VUSs in *CEP290* and one pathogenic variant in *SPATA7* were observed. Both *CEP290* VUSs were inherited from the father, and one VUS (c.4661_4663delAAG) was previously reported as a disease-causing mutation [17], but further validation is needed. Because another VUS 1 bp

upstream of the ATG start site (c.-1G>A) of *CEP290* might affect gene transcription, the pathogenicity of the VUS could not be excluded. Additional sequencing was performed for c.2991+1655A>G, a well-known deep intronic mutation in *CEP290*, but the results were negative. The frameshift variant in *SPATA7* was confirmed to be inherited from the mother. Although the same heterozygous variant was reported as pathogenic in a Chinese patient with LCA with an additional mutation in *GUCY2D* as digenic manner [18], we concluded that this frameshift deletion should be classified as VUS based on the results of maternal inheritance and another likely pathogenic mutation detected in *CEP290* after additional targeted panel sequencing.

In P6 and P8, only a single mutated allele was found in *NMNAT1* and *RPGRIP1*, respectively. Additional copy number analyses using the ExomeDepth algorithm and our preestablished pipeline failed to find any pathogenic deletion or duplication.

Additional targeted panel sequencing with copy number variation analysis in three unsolved patients: We performed additional targeted panel next-generation sequencing analysis with 113 genes responsible for infantile nystagmus syndrome in three unsolved patients (Appendix 1). These 113 genes included two recently discovered genes (*CLUAP1* and *DTHD1*) and are listed in a Appendix 1. In P6, additional analysis found a nonsense mutation c.3946C>T, p.Gln1316Ter in the *RP1L1* gene (Gene ID 94137, OMIM 608581). Because *RP1L1* causes occult macular dystrophy as an autosomal dominant inheritance pattern, this novel nonsense variation is not related to the patient's phenotype. In P7, additional analysis of targeted NGS revealed a new intronic variant c.6012–12T>A *CEP290* was found. This mutation was previously found in Joubert syndrome [19,20] and was classified

TABLE 2. RESULTS OF MUTATION ANALYSIS IN 9 PATIENTS USING TRUSIGHT ONE PANEL AND TARGETED PANEL SEQUENCING.

Patients	Gene	Inheritance	Nucleotide change	Amino acid change	Depth	Zygosity	Origin of mutation	SIFT	ACMG classification	Allele frequency	References
P1	*CRX*	AD	c.442delG	p.Gly148AlafsTer39	40	Hetero	De novo	-	P	Not found	Novel
P2	*GUCY2D*	AR	c.2649delT	p.Phe883LeufsTer13	111	Hetero	Paternal	-	P	Not found	Novel
			c.3038G>A	p.Gly1013Glu	68	Hetero	Maternal	DT(0)	LP	Not found	Novel
P3	*GUCY2D*	AR	c.1991A>C	p.His664Pro	53	Hetero	Paternal	DT(0)	LP	Not found	Novel
			c.2649delT	p.Phe883LeufsTer13	105	Hetero	Maternal	-	P	Not found	Novel
P4	*NMNAT1*	AR	c.196C>T	p.Arg66Trp	81	Hetero	Maternal	DT(0)	LP	0.00008529	2012 Nat Genet [43], 2015 J Biol Chem [44]
			c.709C>T	p.Arg237Cys	30	Hetero	Paternal	DT(0)	LP	0.00005052	2012 Nat Genet [43], 2015 J Biol Chem [44], 2012 Nat Genet [45]
P5	*NMNAT1*	AR	c.196C>T	p.Arg66Trp	96	Hetero	Maternal	DT(0)	LP	0.00008529	2012 Nat Genet [43], 2015 J Biol Chem [44]
			c.709C>T	p.Arg237Cys	48	Hetero	Paternal	DT(0)	LP	0.00005052	2012 Nat Genet [43], 2015 J Biol Chem [44], 2012 Nat Genet [45]
P6	*NMNAT1*	AR	c.709C>T	p.Arg237Cys	25	Hetero	Paternal	DT(0)	LP	0.00005052	2012 Nat Genet [43], 2015 J Biol Chem [44], 2012 Nat Genet [45]
P7	*CEP290*	AR	c.-1G>A	—	110	Hetero	Paternal	-	VUS	0.00003291	Novel
			c.4661_4663delAAG	p.Glu1554del	83	Hetero	Paternal	-	VUS	0.00002529	2007 Hum Mutat [17]
			c.6012-12T>A	—	1252	Hetero	Maternal	-	LP	0.00002369	2013 J Hum Genet [19], 2016 Exp Mol Med [20]
P8	*SPATA7*	AR	c.20_23delTCAG	—	44	Hetero	Maternal	-	VUS	0.0004735	2011 PLoS One [18]
	RPGRIP1	AR	c.3565_3571delCGAAGGC	p.Arg1189GlyfsTer7	163	Hetero	Unknown	-	LP	0.00001625	2008 Mol Vis [41]
P9	*RPGRIP1*	AR	c.2079C>G	p.Tyr693Ter	231	Hetero	Unknown	-	LP	Not found	Novel
			c.2209_2215+18del	—	13	Hetero	Unknown	-	LP	Not found	Novel

Mutations in bolded characters indicate the detection by targeted panel sequencing. Abbreviation: ACMG, American College of Medical Genetics; VUS, variant of unknown significance; AD, autosomal dominant; AR, autosomal recessive; DT, deleterious; Hetero, heterozygous; M, materal; P, paternal.

as a likely pathogenic mutation based on the result of trans-detection for recessive disorder in the trio study. In P8, no additional variants including copy number variation (CNV) were discovered other than the same frameshift mutation in *RPGRIP1*. Because copy number changes might be missed by exome-depth algorithms, multiplex ligation-dependent probe amplification (MLPA) assay for *RPGRIP1* using the SALSA MLPA P222 LCA kit (MRC Holland, Amsterdam, the Netherlands) was performed. However, the assay also failed to discover any deletion or duplication, including the exon 17 deletion previously reported in Japanese patients with LCA [21].

Genotype-phenotype correlation: Demographic and clinical features of the enrolled patients are summarized in Table 3. None of the nine patients had syndromic features such as hearing loss, renal failure, or gross motor delay. All patients were babies around 1 year of age except P9 who was advised for genetic testing at the age of 29 years. Considering the association between affected genes and fundus findings, the patients carrying *GUCY2D* mutations (P2 and P3) showed grossly normal retinal appearances without noticeable retinal vessel caliber changes or pigmentary changes. In addition, patients carrying *NMNAT1* mutations (P4, P5, and P6) displayed macular coloboma-like atrophic lesions and retinal vessel narrowing that were discovered in early infancy (Figure 1). Although we found only a single pathogenic variant in *NMNAT1* (p.Arg237Cys) in P6, the retinal examination of that patient showed a typical macular coloboma-like lesion in early infancy. None of these patients had overt optic atrophy at the time of examination.

No developmental delay or systemic abnormalities were found in the patient with suspected *CEP290* mutations (P7). This 7-month-old female had oculodigital sign and wandering eye movement. The retinal examination showed a normal optic disc, no pigmentary retinopathy, and only mild vessel narrowing.

Interestingly, P9 was initially misdiagnosed with idiopathic infantile nystagmus because the initial fundus finding was grossly normal in early infancy. The patient underwent Anderson-Kestenbaum surgery at the age of 6 years for significant left head turn. His best-corrected visual acuity remained 20/200 bilaterally. At the age of 29 years, targeted NGS revealed a compound heterozygous *RPGRIP1* mutation in this patient. Dilated fundus examination showed mild vessel attenuation and temporal optic atrophy without overt retinal pigmentary changes. Optical coherence tomography showed a relatively preserved inner segment and outer segment line (Figure 2).

The c.709C>T, p.Arg237Cys variant in the *NMNAT1* gene is known as a pathologic variant. This variant was found in all three individuals with macular coloboma-like degeneration, including one heterozygous individual (Figure 3). The allele frequency is 0.000074 (nine alleles out of 121,284 alleles are reported) in the ExAC browser and 0.00031 (two alleles out of 6,501 alleles) in the Exome Variant Server. However, the c.709C>T allele frequency was 0.0024 in the Korean Reference Genome Database (KRGDB), which consists of the whole exome sequencing results for 622 Korean individuals, suggesting that this mutation is relatively common in the Korean population.

DISCUSSION

Several custom NGS panels [22,23] and commercial panels [24] have been reported to show high diagnostic utility in patients with LCA. We evaluated a commercial clinical exome kit that encompasses 4,813 genes associated with known clinical phenotypes, including 22 genes associated with LCA. The panel also includes genes for other retinal disorders and can help make a differential diagnosis. For example, differentiation of LCA from achromatopsia or syndromic retinal disorders is practically impossible, especially in young infants. These disease entities not only present similar phenotypes in early infancy but also genetically overlap each other (e.g., mutations in *CEP290* cause either Joubert syndrome or LCA). Another strength of using commercial kits is the feasibility of standardization and inter-laboratory harmonization. However, the list of disease-related genes is rapidly expanding [15], which suggests that using a fixed set could miss candidate genes. Therefore, new genes like *CLUAP1* can be missed with a clinical exome kit that targets already known genes that cause LCA. Another drawback is that targeting too many genes could lead to a reduced average depth of coverage and more regions with low depth. In a missense *RPGRIP1* variant (P9), the depth of coverage was 13, and further confirmation with Sanger sequencing was needed.

LCA can be diagnosed at an early age with careful clinical examination and ERG testing. Considering the progressive nature of retinal degeneration in LCA, early molecular diagnosis could open up an opportunity to initiate a gene therapy trial [25]. Although still in clinical trials, recent gene therapy studies have shown that great improvements in visual acuity can be achieved in young patients with better baseline visual acuity [26]. Initial phase 1 and 2 trials of a gene therapy for *RPE65* have been accomplished [27], and those for *GUCY2D* and gene editing for *CEP290* are under investigation [28,29].

TABLE 3. DEMOGRAPHICS AND CLINICAL FEATURES OF PATIENTS SUSPECTED WITH LEBER CONGENITAL AMAUROSIS.

Patients	Sex	Age at testing	Gene	Main symptom	Fundus finding	ERG	Oculodigital sign	Cycloplegic refraction
P1	F	7.5 months	*CRX*	Poor eye contact	Grossly normal, OU	Extinguished	+	OD: +sph3.00 OS: +sph2.50
P2	M	6 months	*GUCY2D*	Poor eye contact	Grossly normal, OU	Extinguished	-	OD: +sph5.50 OS: +sph5.50
P3	F	6 months	*GUCY2D*	Nystagmus Poor eye contact	Grossly normal, OU	Extinguished	-	OD: +sph8.50 OS: +sph8.50 -cyl100 axis180
P4	M	13 months	*NMNAT1*	Eye poking, rubbing	Bony speculated pigmentation Macular coloboma, OU	Extinguished	+	R: +sph5.50 L: +sph5.50
P5	F	7 months	*NMNAT1*	Poor eye contact	Bony speculated pigmentation Macular coloboma, OU	Extinguished	+	R: +sph4.00 L: +sph5.00
P6	M	12 months	*NMNAT1*	Nystagmus	Macular coloboma Marbled fundus, OU	Extinguished	+	OD: +sph5.50 -cyl3.00 axis180 OS: +sph5.00 -cyl3.00 axis180
P7	F	7 months	*CEP290*	Poor eye contact	Grossly normal, OU	Extinguished	+	OD: +sph6.00 OS: +sph6.00
P8	F	8 months	*RPGRIP1*	Poor eye contact	Pale disc, OU	Extinguished	-	OD: +sph6.00 -cyl0.75 axis170 OS: +sph6.00 -cyl0.50 axis10
P9	M	29 years	*RPGRIP1*	Nystagmus	Pale disc, OU	Extinguished	-	OD: -sph6.50 OS: -sph6.50

Abbreviation: ERG, electroretinogram, OD, oculus dexter; OS, oculus sinister, OU, oculus uterque
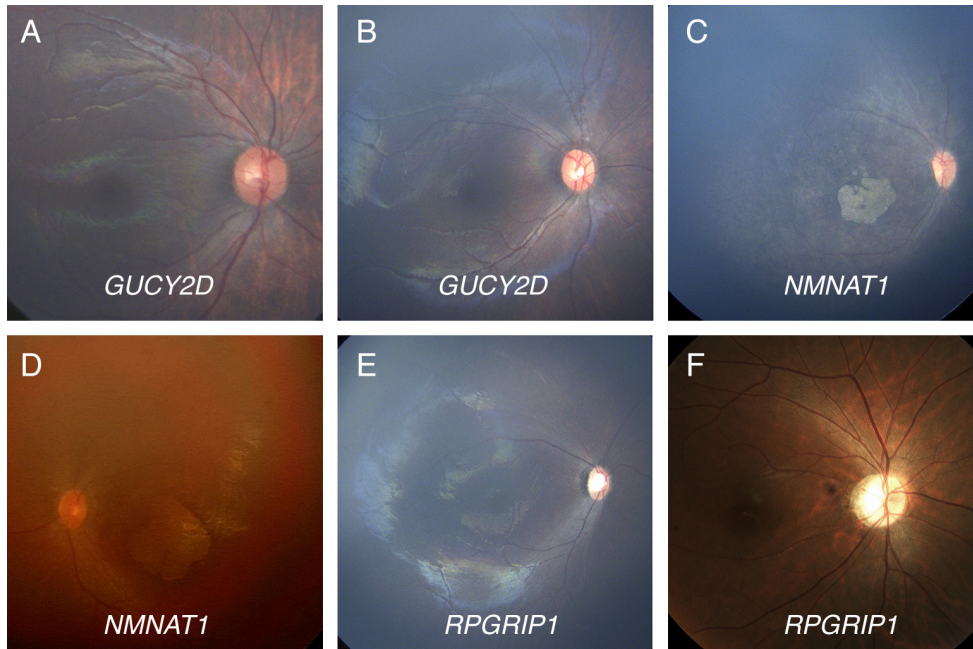
Figure 1. Color photographs of the retinas of six patients with LCA. **A**, **B**: Two 6-month-old babies (P2 and P3) with compound heterozygous mutations in *GUCY2D* (p.[Phe993LeufsTer13]+[Gly1013Glu] and p.[His664Pro]+[Phe883LeufsTer13], respectively) have no definite abnormal finding (OD) in the retina. **C**: A 7-month-old baby (P5) with compound heterozygous missense mutations in *NMNAT1* (p.[Arg66Trp]+[Arg237Cys]) has marked chorioretinal atrophic changes in the macula with vessel narrowing and mild pigmentary changes (OD). **D**: A 1-year-old baby (P6) with only one pathogenic mutation in *NMNAT1* (p.[Arg237Cys]+[?]) shows nystagmus, hyperopia, and a characteristic macular coloboma-like lesion (OS). **E**: An 8-month-old baby (P8) presented with poor eye contact, extinguished electroretinogram, +6.00 diopters hyperopia, slight optic disc pallor, and mild vessel narrowing (OD). We identified one frameshift mutation (p.Arg1189GlyfsTer7) in *RPGRIP1*. **F**: A 29-year-old male (P9) presented with nystagmus developed at the age of 6 years, 20/200 acuity, optic atrophy, and mild vessel attenuation (OD). Compound heterozygous mutations were identified in *RPGRIP1* (p.[Tyr693Ter]+[c.2212_2215+21del]).

Genotype–phenotype correlation is important in narrowing down causative genes, particularly in genetically heterogeneous disorders. The present study showed that all three patients with coloboma-like macular atrophic lesions had *NMNAT1* mutations, whereas those with grossly normal retinal appearances had mutations in *GUCY2D*, *CRX*, and *CEP290*, consistent with previous reports [30,31]. The other types of LCA with macular atrophic lesions present in infancy or later in life have been reported to occur in individuals carrying mutations in *AIPL1*, *CRB1*, *RDH12*, *RPGRIP1*, *TULP1*, and *RPE65* [32-34]. Those other types show progressive macular degeneration in the natural course, whereas macular coloboma-like degeneration in early infancy could be specific to mutations in *NMNAT1*. Therefore, single-gene sequencing of *NMNAT1* could be a cost-effective strategy in
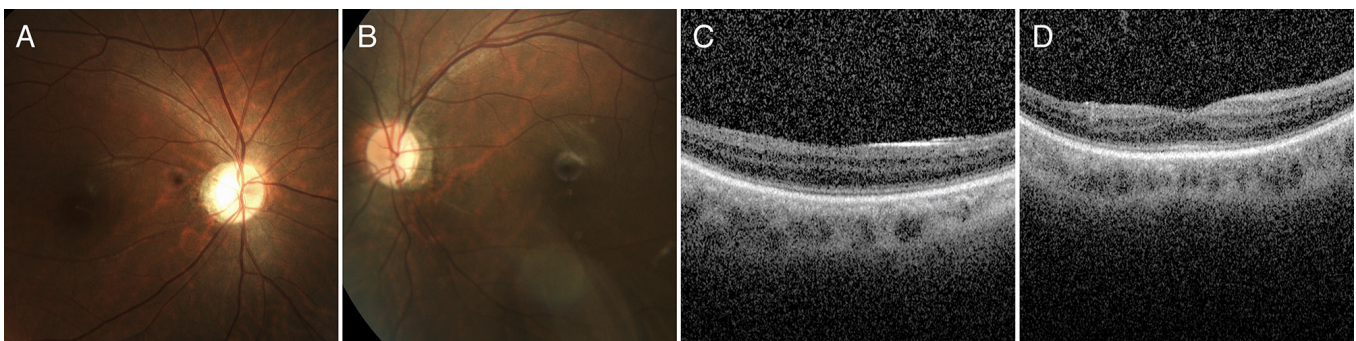

Figure 2. Fundus photographs of a 29-year-old male with mutations in *RPGRIP1* who was initially misdiagnosed with idiopathic infantile nystagmus. He had sustained left head turn posture with left beating jerk nystagmus. Anderson-Kestenbaum surgery was performed at the age of 6 years. At the age of 29 years, his best visual acuity was 20/200 bilaterally. **A**, **B**: Fundus photography shows mild optic atrophy with retinal vessel attenuations. **C**, **D**: Spectral domain optical coherence tomography shows thinning of the inner segment and outer segment line.
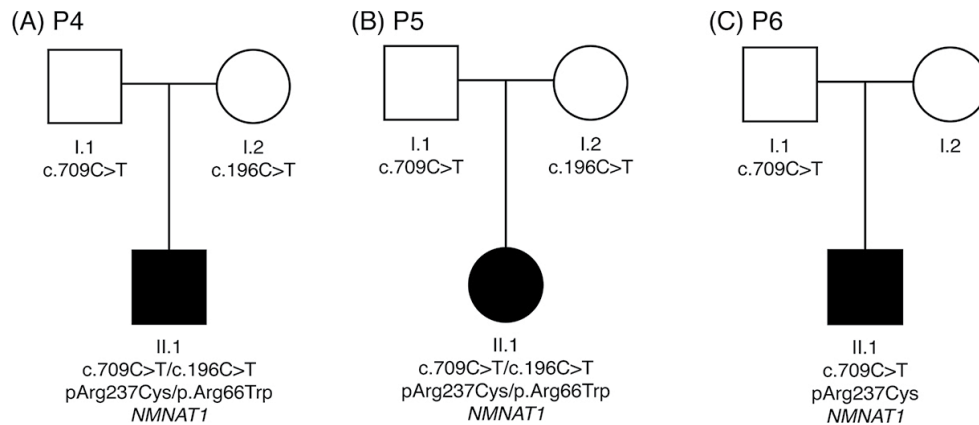
Figure 3. Pedigrees of three patients with mutations in *NMNAT1*. These patients were non-consanguineous, and all patients harbored a pathogenic mutation in *NMNAT1* (c.709C>T; p. Arg237Cys), which was supposed to be a founder mutation in Koreans.

patients with macular coloboma-like degeneration in early infancy, especially when NGS testing is unavailable.

One patient with mutations in *RPGRIP1* (P9) was initially misdiagnosed with an idiopathic form of infantile nystagmus, but the diagnosis changed because of the NGS results. Most patients with mutations in *RPGRIP1* have a grossly normal fundus in early infancy [35]. Khan et al. reported a 6-year-old girl with a mutation in *RPGRIP1* whose nystagmus abated when she adapted a moderate right face turn [35]. Thus, careful clinical examination, including of the peripheral retina, is essential in the diagnosis of LCA, and meticulous ERG testing should be conducted in patients who exhibit poor visual acuity.

One patient (P7) had two heterozygous VUSs in *CEP290*, of paternal origin, and one heterozygous pathogenic variant in *SPATA7* of maternal origin. It is possible that mutations in those genes work together as "digenic inheritance" [36]. However, additional targeted panel sequencing revealed another intronic variant in *CEP290* that could be classified as likely pathogenic and was confirmed to be inherited from the mother. Although LCA has been known to be inherited as digenic pattern in some reports [36-38], it is important to be cautious about the possibilities of a digenic inheritance because genetic testing might miss mutations in the affected gene.

Several genetic studies of patients with LCA have been performed in different ethnic groups [39,40]. Seong et al. [22,41,42] performed consecutive genetic studies on a few genes in Korean patients with LCA but failed to find any founder mutation. Through comprehensive testing on several genes, we found three common alleles associated with LCA: *GUCY2D* c.2649delT, *NMNAT1* c.196C>T, and *NMNAT1* c.709C>T. *NMNAT1* was only recently identified as a causative gene for LCA. The minor allele frequency of *NMNAT1* c.709C>T (p.Arg237Cys; rs375110174) is estimated to be as

high as 0.24% (Korean Reference Genome DB). Therefore, mutations in *NMNAT1* and *GUCY2D* might be major genes responsible for LCA in Koreans, which contrasts with the high frequency of mutations in *CEP290* in Caucasian populations [2]. Additionally, one frameshift mutation found in *RPGRIP1* in P8 has also been reported in another Korean patient [41].

In conclusion, the present study supports the utility of clinical exome sequencing in LCA diagnosis and differential diagnosis from other syndromic diseases with overlapping ocular phenotypes. Understanding the technology, applying careful interpretation, supplementing with other testing, and correlating with clinical phenotypes could lead to the successful implementation of clinical exome sequencing in the routine diagnosis of inherited retinal disorders, such as LCA.

APPENDIX 1. TARGET GENES WERE LISTED.

To access the data, click or select the words "Appendix 1." Genes included in infantile nystagmus syndrome target enrichment. Listed are the genes included in the custom designed target enrichment along with the disease or phenotype associated with the gene according to Online Medelian Inheritance in Man (OMIM), OMIM phenotype identification number, and OMIM or Gene Cards gene identification number. Genes are named according HUGO Gene Nomenclature Committee (HUGO, http://www.genenames.org/) approved nomenclature.

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