

A Homozygous Missense Mutation in *NEUROD1* Is Associated With Nonsyndromic Autosomal Recessive Retinitis Pigmentosa

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PURPOSE. Mutations in the same gene can lead to different clinical phenotypes. In this study, we aim to identify novel genotype-phenotype correlations and novel disease genes by analyzing an unsolved autosomal recessive retinitis pigmentosa (ARRP) Han Chinese family.

METHODS. Whole exome sequencing was performed for one proband from the consanguineous ARRP family. Stringent variants filtering and prioritizations were applied to identify the causative mutation.

RESULTS. A homozygous missense variant, c.724G>A; p.V242I, in *NEUROD1* was identified as the most likely cause of disease. This allele perfectly segregates in the family and affects an amino acid, which is highly conserved among mammals. A previous study showed that a homozygous null allele in *NEUROD1* causes severe syndromic disease with neonatal diabetes, systematic neurological abnormalities, and early-onset retinal dystrophy. Consistent with these results, our patients who are homozygous for a less severe missense allele presented only late-onset retinal degeneration without any syndromic symptoms.

CONCLUSIONS. We identified a potential novel genotype-phenotype correlation between *NEUROD1* and nonsyndromic ARRP. Our study supports the idea that *NEUROD1* is important for maintenance of the retina function and partial loss-of-function mutation in *NEUROD1* is likely a rare cause of nonsyndromic ARRP.

Keywords: retinitis pigmentosa, *NEUROD1*, genotype-phenotype correlation, next-generation sequencing, retina

It has been observed that different mutations in the same gene can lead to distinct clinical phenotypes in human patients. This is particularly common for genes that play important roles during multiple developmental stages and/or in different tissues. In these cases, complete loss-of-function (LOF) mutations often cause syndromic phenotypes involving multiple organ systems or showing earlier onset of disease, while partial LOF mutations might lead to a milder phenotype or a subset of the phenotypes caused by complete LOF mutations. For example, human *CLN3* plays an important role in lysosome function in brain and retina. Homozygous deletion of a 1.02 kb genomic region, including two coding exons, in *CLN3* gene is a major cause of juvenile neuronal ceroid lipofuscinoses, which is characterized by early-onset photoreceptor degeneration, severe neurological impairment, and premature death.¹ By contrast, missense mutations in *CLN3* can cause late-onset retinal degeneration without any syndromic involvements.² Uncovering these phenotype-genotype correlations is of

clinical importance for molecular diagnosis. Furthermore, a mutation spectrum with corresponding phenotypes can provide structural and functional insights about this gene/protein.

NEUROD1, also known as neurogenic differentiation factor 1, is a basic helix-loop-helix (bHLH) transcription factor. Human patients with a homozygous frameshift mutation in *NEUROD1* developed early-onset diabetes and neurological abnormalities including retinal dystrophy, suggesting *NEUROD1* plays an essential role in early development.³ *Neurod1* deficient mice also show severe neonatal diabetes.⁴ By contrast, the retinal degeneration in *Neurod1* knockout mice is relatively late-onset indicating that *Neurod1* is also required for adult photoreceptors maintenance.^{5,6} These findings highlighted the potential diverse function of *NEUROD1* in multiple tissues and at different stages.

In this study, we identified a homozygous missense mutation in *NEUROD1* associated with nonsyndromic retinitis pigmen-

tosa in a consanguineous family by whole exome sequencing (WES). This finding suggests *NEUROD1*'s essential function in maintaining adult photoreceptors in human and also supports the hypothesis that diverse clinical phenotypes can be caused by mutations in the same gene.

MATERIALS AND METHODS

Clinical Diagnosis of RP

Written consent was obtained from all participating individuals. The study was approved by the Institutional Review Board of Peking Union Medical College Hospital (PUMCH), and adhered to the Declaration of Helsinki. Blood samples were obtained from five family members, including two affected siblings, and DNA was extracted using QIAamp DNA Blood Mini Kit as instructed by the manufacturer (Qiagen, Hilden, Germany). The proband was identified at the Ophthalmic Genetic Clinic, PUMCH in Beijing, China. Detailed ophthalmic evaluations included best-corrected visual acuity (BCVA) tests, slit-lamp biomicroscopy, dilated indirect ophthalmoscopy, fundus photography, and visual field tests (Octopus; Interzeag, Schlieren, Switzerland). Optical coherence tomography (OCT; Topcon, Tokyo, Japan) was applied to scan the retina structure. Electroretinography (ERG) with corneal "ERGjet" contact lens electrodes was carried out (RetiPort ERG system; Roland Consult, Wiesbaden, Germany). The ERG protocol complied with the standard published by the International Society for Clinical Electrophysiology of Vision (www.iscev.org, provided in the public domain by the International Society for Clinical Electrophysiology of Vision).

Library Preparation and WES

The Illumina paired-end DNA library (Illumina, San Diego, CA, USA) preparation followed the standard manufacturer's instruction. Briefly, DNA was quantified using NanoDrop (Thermo Scientific, Wilmington, DE, USA). One microgram of total DNA was sheared into fragments of 300 to 500 bp, end-paired, and had a 3' Adenosine base added. Then Illumina Y-shaped adapters were added to the DNA fragments, and 10 cycles of PCR reactions were applied to amplify the libraries. The library DNA was further quantified using a picogreen assay. Six library DNA samples were pooled together before the capture step. In total 3 µg of pooled DNA was enriched by the NimbleGen SeqCap EZ Hybridization and Wash kit (Nimblegen SeqCap EZ Human Exome Library version 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.^{2,7,8} In particular, raw variants were called using Atlas2 Suite.⁹ Considering the rareness of RP, variant with a frequency of >0.5% in any of the variant databases queried, including 1000 Genome,¹⁰ dbSNP135,¹¹ National Heart, Lung, and Blood Institute Exome Sequencing database,¹² National Institute of Environmental Health Sciences Exome Sequencing database,¹³ an internal control database of 11,000 exomes, and an ethnicity-matched control database (approximately 4000 exomes) from Exome Aggregation Consortium (ExAC)¹⁴ was filtered out. dbNSFP version 2.3 was used to functionally predict the effects of missense variants.^{15,16}

Validation of Variants by Dideoxy Sequencing

For each identified mutation, a 500-bp flanking sequence at both sides was obtained from the University of California, Santa Cruz, genome browser (hg19 assembly). RepeatMasker was used to mask the repetitive sequence in human genome.¹⁷ 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.¹⁸ After PCR amplification, the amplicons were sequenced on an ABI 3730xl or 3500XL Genetic Analyzer (Life Technologies, Carlsbad, CA, USA).

RESULTS

Clinical Diagnosis Revealed a Typical Adult-Onset RP Phenotype With No Syndromic Involvements

The pedigree is originated from Liaoning province of Northern China. All the family members are Han Chinese. The pedigree shows a typical autosomal recessive inheritance pattern with third-degree consanguinity (first-cousins; Fig. 1a). The proband was a 33-year-old male complaining about night blindness for 15 years and exhibited a progressive decline in visual acuity for 8 years. His BCVA was 0.6 (Snellen decimal chart) for both eyes. Slit-lamp examination revealed no obvious abnormalities of the anterior segment. His fundus showed bone-spicule pigmentation in the mid-periphery region with attenuated retinal vessels (Figs. 2a, 2b). Optical coherence tomography illustrated increased retinal thickness in the macular region with discontinuous inner segments/outer segments junction signal, and loss of foveal pit (Figs. 2e, 2f). Visual field test showed symmetrically loss of upper and temporal visual field with conserved central visual field in both eyes (Supplementary Fig. S1). Electroretinography displayed significantly reduced amplitudes and prolonged implicit time for both rod and cone reactions, which reflected widespread rod and cone degeneration (Supplementary Fig. S2). The proband's affected elder sister was 40 years old and suffered night blindness since childhood. Her BCVA was 0.1/0.15 (right/left). The slit-lamp examination revealed severe subcapsular cataract in both eyes. Diffused retinal pigment epithelial and choroidal atrophy with characteristic bone-spicule pigmentation in the mid-periphery was recorded (Figs. 2c, 2d). Vitreous membrane was observed in her right eye. No clear OCT image was obtained due to her cataract. No systemic abnormality was found for both patients. All of the ophthalmic and neurological clinical data was taken at ages 33 and 40, respectively. Detailed clinical phenotypes of the two affected members are listed in the Table.

Whole Exome Sequencing Identified a Homozygous Variant c.724G>A; p.V242I in *NEUROD1* as the Top Candidate Disease-Causing Mutation

As a prescreening step, customized capture sequencing of 186 known retinal disease-causing genes was performed for the male proband as described before.² No clear pathogenic mutations in any known retinal disease genes were identified, suggesting mutations in a novel gene might be the cause of disease. Whole-exome capture sequencing of the male proband was then performed to identify the potential novel disease gene. A total of 56.6 million reads were generated, 97.8% of which were mapped to human reference genome hg19, achieving a mean coverage of 59X across the whole exome. Over 180,000 different variants were initially called by Atlas suite.⁹ After extensive filtering and annotation to exclude common polymorphisms,^{2,8} only 549 rare and coding-change

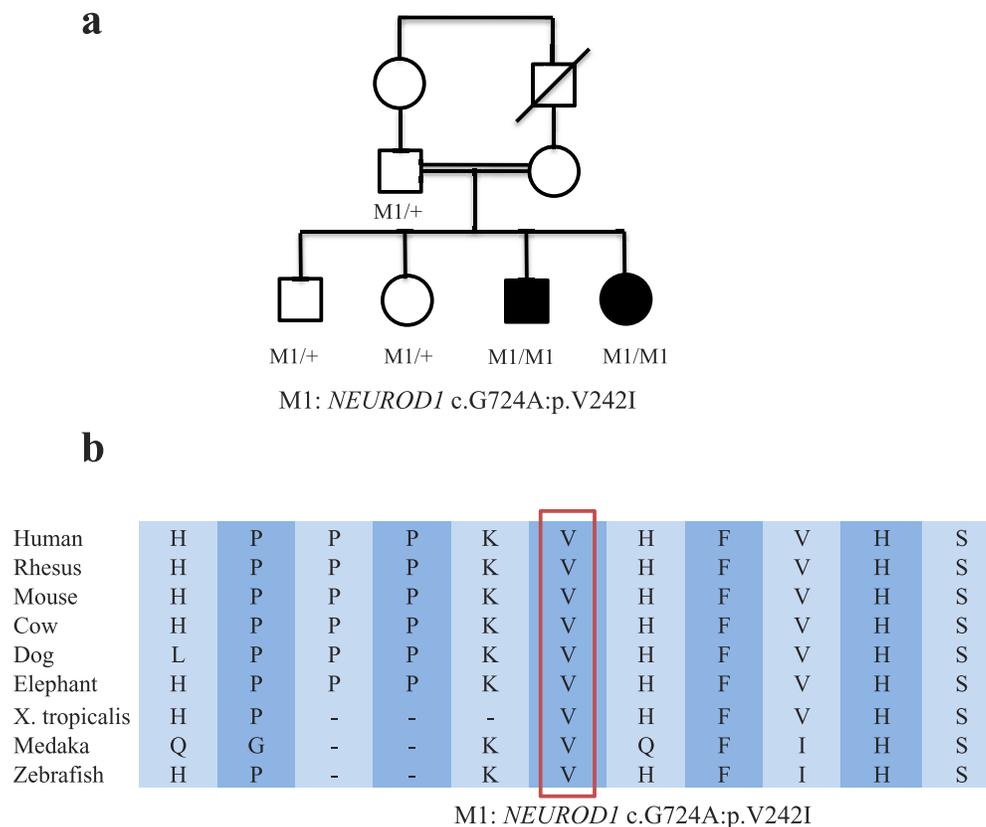


FIGURE 1. (a) Pedigree of the consanguineous ARRP family. The V242I mutation in *NEUROD1* segregates perfectly among the family. (b) Multispecies alignment around the amino acid affected by the V242I mutation in *NEUROD1*. The affected amino acid is highly conserved among mammals.

variants remained. Among them, 16 were in the homozygous state, and 14 were in compound heterozygous state.

Considering the fact of consanguinity, we first focused on the 16 homozygous variants and performed a series of exclusions and prioritizations based on integrative analysis of various sources of information in order to identify the causative mutation (Fig. 3; Supplementary Table S1). Specifically, four variants on chromosome X were given low priority as the inheritance model is not likely to be X-linked. Two variants were excluded because homozygous LOF variants in the same genes were observed in multiple control samples. Two variants were excluded because previous studies indicated recessive mutations in the same genes did not cause retinal phenotype in humans. Three variants were considered low priority since no retinal phenotype was reported in the knockout mouse model of the respective genes. Examination of human RNA-seq data showed four additional variants occurred in genes with little or no expression in the retina making it unlikely they can cause retinal disease.¹⁹ In addition, we also performed the same exclusions and prioritizations for all 14 compound heterozygous variants and found no promising candidates (Supplementary Table S2).

As a result, a homozygous missense variant (NM_002500: c.724G>A; p.V242I) in *NEUROD1* was identified as the top candidate disease-causing mutation. The mutation was never found in any control database with a total of over 20,000 samples (1000 Genome, ESP6500, internal exome sequencing database of 11,000 individuals, 4000 ethnicity-matched control exomes from ExAC).^{10,12} It affects amino acid, which is highly conserved across mammals (Fig. 2b), and has a mixture of prediction scores ranging from “Damaging” to “Benign” according to different *in silico* prediction algorithms (Supple-

mentary Table S3). This mutation is validated by direct Sanger sequencing. Furthermore, segregation test of this mutation with RP was performed. Both affected individuals are homozygous for this mutation, while all unaffected family members are either heterozygous for the allele or wild type (Fig. 1a).

DISCUSSION

NEUROD1 is a bHLH transcription factor. It is highly expressed in many tissues including the retina, brain, and pancreas. It binds to E box-containing promoter sequences to serve as a transcription activator, thus specifically regulating gene expression.^{20,21} *NEUROD1* has been shown to play critical roles in multiple biological processes including endocrine cell development, neurogenesis, and glucose homeostasis.²²⁻²⁶

Several reports associate *NEUROD1* mutations with human genetic disease, though almost all of these reports have focused on diabetes. Heterozygous LOF mutations in *NEUROD1* can cause autosomal dominant type 2 diabetes.²⁷ A number of studies also linked *NEUROD1* variants to diabetic cohorts.²⁸⁻³¹ Strikingly, a single-family report showed that a homozygous frameshift mutation in *NEUROD1* can cause a syndromic phenotype that includes retinal dystrophy, permanent diabetes with onset within the first 2 months of life, and early-onset systematic neurological abnormalities.³ This strongly suggests *NEUROD1*'s essential role in multiple tissues, including retina. Hence, the complex genotype-phenotype correlations of *NEUROD1* mutations and its potential diverse roles should not be ignored.

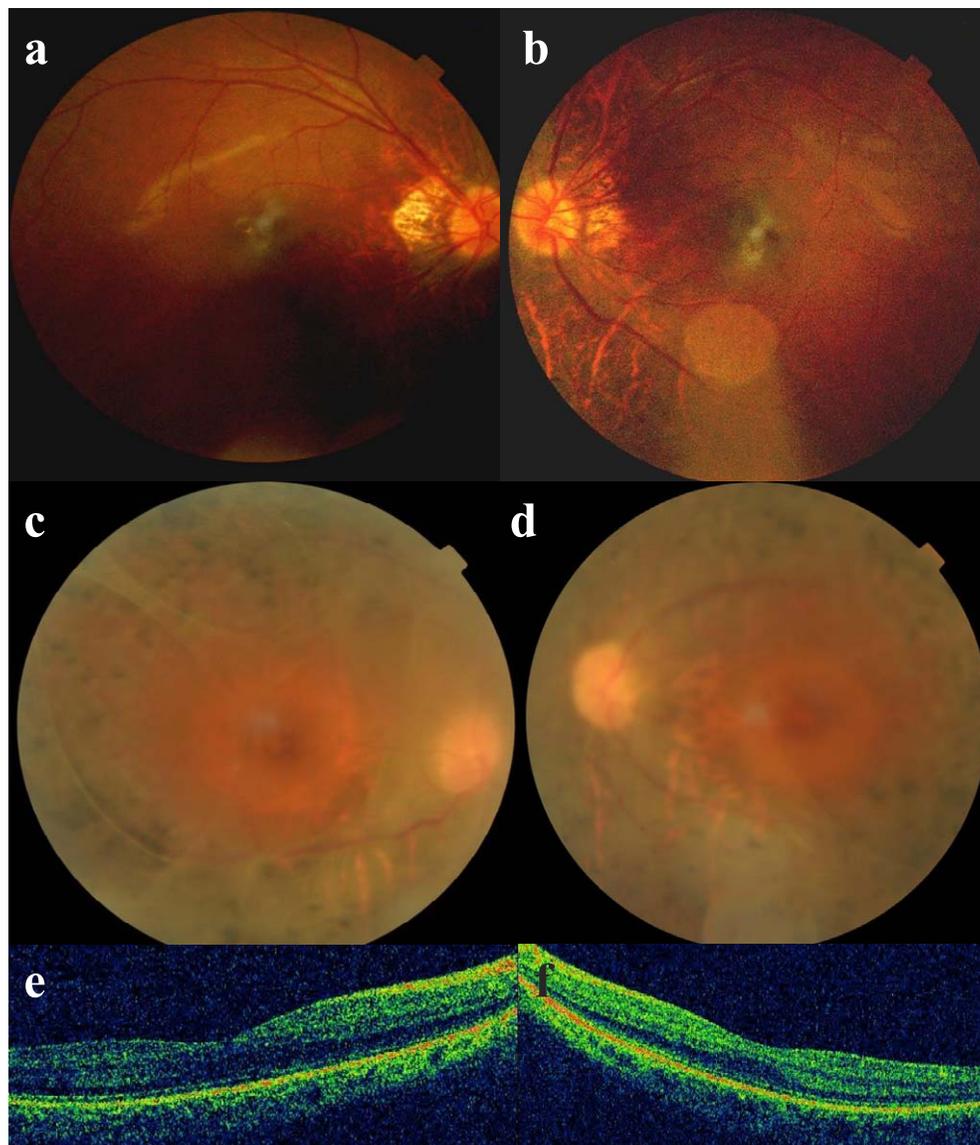


FIGURE 2. Fundus photograph and OCT of the affected family members. Right (a) and left (b) fundus of the proband. Widespread retinal pigment epithelial and choroidal atrophy with pigments in the inferior periphery fundus was observed. Right (c) and left (d) fundus of the proband's elder sister. Defused retinal pigment epithelial and choroidal atrophy with typical bone-spicule pigmentation in the mid-periphery was observed. Vitreous membrane was observed in her right eye. Right (e) and left (f) eye OCT of the proband. Loss of foveal pit with increased retinal thickness in the macular region was observed. The inner segments/outer segments signal was discontinuous.

Consistent with the phenotype observed in human patients, *Neurod1*^{-/-} mice under C57BL/6 background display severe neonatal diabetes and die within 5 days after birth due to defects in β cell differentiation.⁴ Interestingly, *Neurod1*^{-/-} mice under 129SvJ background have higher survival rate and mice overcoming early lethality show progressive photoreceptor degeneration, suggesting *Neurod1*'s important function for adult photoreceptor maintenance.⁵ This hypothesis is further supported by a conditional knockout of *Neurod1* in mouse retina. Using a *Crx* promoter, this study confirmed that *Neurod1* is required for long-term photoreceptor maintenance and loss of *Neurod1* results in age-related degeneration of both rod and cone photoreceptors.⁶

In our consanguineous ARRP family, both patients were diagnosed as late-onset RP and did not display any other abnormalities despite a complete clinical exam and thorough testing for diabetes and neurological disorders (Table). One explanation for this milder phenotype is that the missense

allele carried by the patient is a partial LOF mutation. Given that the patients do not have developmental defects and any other syndromic features, the mutation might only affect the function critical for maintenance of the adult photoreceptor cell. Consistent with this interpretation, the mutation is not located within the bHLH functional domain, which is critical for the protein's function in early developmental process. Therefore, these results suggest that LOF mutations in *NEUROD1* cause severe early-onset syndrome while less-damaging missense mutations cause late-onset nonsyndromic retinal degeneration. In order to strengthen this conclusion, we screened over 300 unsolved RP cases for homozygous or compound heterozygous mutations in *NEUROD1*. Unfortunately, no additional supporting cases were observed. With only one pedigree and no functional data, any conclusions should be tentative. However, given all the genetic and functional evidences we have presented above, we believe that the V242I mutation in *NEUROD1* is the probable cause of

TABLE. Clinical Features of Patients With *NEUROD1* Mutations

	Proband	Proband's Affected Sister
Sex/age	M/33*	F/40*
Age of onset	18	10
BCVA, OD/OS	0.6/0.6	0.1/0.15
Anterior segment	NA	Dense subcapsular cataract
Fundus	Bone-spicule pigmentation in the mid-periphery	Diffused RPE and choroidal atrophy with bone-spicule pigmentation
OCT	Increased retinal thickness in the macular region with discontinuous IS/OS junction	No clear image because of cataract
VF	Loss of upper and temporal visual field	NA
ERG	Moderately decreased rod and cone responses	Nonrecordable
BMI	20.2	18.4
FBG, mmol/L	5.4	5.1
HbA1c, %	6.5	6.2
Neurological abnormality		
Developmental delay	No	No
Ataxia	No	No
Sensorineural deafness	No	No
Seizure	No	No

VF, visual field; BMI, body mass index; FBG, fasting blood glucose; HbA1c, glycosylated hemoglobin.

* All of the ophthalmic and neurological clinical data were taken at age 33 and 40, respectively. BMI, FBG, and HbA1c were taken 2 years later.

disease in this consanguineous ARRP family. In addition, as the mechanism for *NEUROD1* in photoreceptors maintenance is not well understood, the identification of this retinal-specific disease allele is an ideal research target for further studies on *NEUROD1*'s role in retina's long-term survival.

In conclusion, we report, for the first time, that a missense mutation in *NEUROD1* is associated with nonsyndromic RP. It is supported by convincing human genetics evidence, obtained

through high-quality next generation sequencing, filtering, and segregation. The results are also consistent with previous reports of a *Neurod1* mouse knockout model, which shows that *Neurod1* is required for survival of adult photoreceptor cells and loss of *Neurod1* causes progressive retinal degeneration.^{5,6} Inclusion of *NEUROD1* in genetic diagnosis panels for patients with nonsyndromic or syndromic retinal degenerative

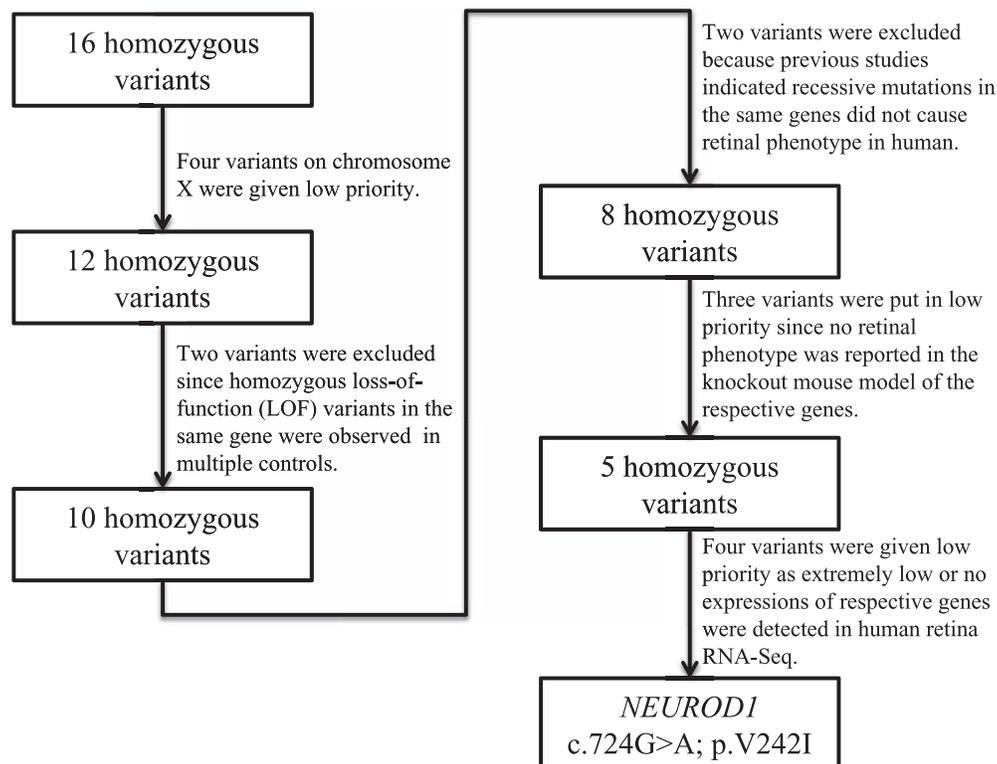


FIGURE 3. Flow chart showing that a series of exclusions and prioritizations based on integrative analysis of various sources of information was performed on 16 homozygous variants.

disease will improve the molecular diagnosis and increase our understanding of *NEUROD1*'s role in the retina.

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