Molecular Vision 2024; 30:58-66 <http://www.molvis.org/molvis/v30/58>
Received 9 July 2023 | Accepted 17 February 2024 | Published 19 February 2024

Two novel non-coding single nucleotide variants in the DNase1 hypersensitivity site of *PRDM13* causing North Carolina macular dystrophy in Korea

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Purpose: Pathogenic variants in North Carolina macular dystrophy (NCMD) have rarely been reported in the East Asian population. Herein, we reported novel variants of NCMD in 2 Korean families.
Methods: The regions associated with NCMD were analyzed with genome sequencing, and variants were filtered based on the minor allele frequency (0.5%) and heterozygosity. Non-coding variants were functionally annotated using multiple computational tools.
Results: We identified two rare novel variants, chr6:g.99,598,914T>C (hg38; V17) and chr6:g.99,598,926G>A (hg38; V18) upstream of *PRDM13* in families A and B, respectively. In Family 1, Grade 2 NCMD and a best-corrected visual acuity of 20/25 and 20/200 in the right and left eyes, respectively, were observed. In Family B, all affected individuals had Grade 1 NCMD with characteristic confluent drusen at the fovea and a best-corrected visual acuity of 20/20 in both eyes. These two variants are 10–22 bp downstream of the reported V10 variant within the DNase1 hypersensitivity site. This site is associated with progressive bifocal chorioretinal atrophy and congenital posterior polar chorioretinal hypertrophy and lies in the putative enhancer site of *PRDM13*.
Conclusion: We identified two novel NCMD variants in the Korean population and further validated the regulatory role of the DNase1 hypersensitivity site upstream of *PRDM13*.

North Carolina macular dystrophy (NCMD/MCDR1, OMIM 136550) is an inherited autosomal dominant retinal disease first described in the 1970s as "dominant macular degeneration and aminoaciduria" by Lefler, Wadsworth, and Sisbury [1]. This disorder has been characterized by macular dystrophy since birth. The clinical phenotype varies within the same family; however, the associated gene exhibits complete penetrance. Fundus findings in NCMD vary, ranging from a small patchy drusen at the fovea (Grade 1) to a large confluent colobomatous concave macular lesion with a thick, white fibrotic rim (Grade 3). The visual acuity in NCMD correlates with the size of the macular lesion and the structural grading of NCMD. Nonetheless, some patients have relatively good vision despite severe macular malformations.

The genetic loci in NCMD were initially mapped to Chromosome 6 by Small et al. [2–4], and the causative variants were identified 15 years after the discovery of the genetic loci [5]. The causative variants were in an intragenic region of Chromosome 6, approximately 13,000 base pairs upstream from the neighboring gene. Currently, 16 NCMD-associated genetic variants have been identified (Appendix 1). Eleven single-nucleotide variants (SNVs) and five tandem duplications were identified as causative variants of NCMD affecting the expression of *PRDM13* (MCDR1 locus) and *IRX1* (MCDR3 locus). The variants upstream of *PDRM13*

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affect the expression of its retinal transcription factor. Most reported variants have been discovered in various ethnicities, including those from Europe, North America, and Brazil. Although V3 mutation and tandem duplication have been reported in Asian families [6,7], the genetic architecture of NCMD in the East Asian population remains relatively unknown. Herein, we investigated the molecular causes of NCMD in Korea and the functional annotation of possible pathogenic variants upstream of *PRDM13* to understand the genetic architectures of molecular causes of NCMD in Korea.

METHODS

Clinical assessment and phenotyping: Two Korean families diagnosed with NCMD were recruited from Gangnam Severance Hospital (Seoul, South Korea) and Seoul National University Bundang Hospital. Clinical assessments included visual acuity testing, dilated fundus examination, wide-field fundus imaging using the Silverstone system (Optos PLC, Dunfermline, Scotland, UK), fundus autofluorescence imaging, and Spectralis optical coherence tomography (OCT, Heidelberg Engineering, Heidelberg, Germany). Informed written consent was obtained from all patients. This study was approved by the institutional review board of Gangnam Severance Hospital (3–2020–0063) and adhered to the tenets of the Declaration of Helsinki.

Genome sequencing (GS) and segregation analysis: Following exome sequencing or targeted-panel next-generation sequencing, two families with undiagnosed NCMD-affected index individuals were subjected to GS. Briefly, samples were prepared according to the Illumina TruSeq DNA PCR-free library preparation (Illumina, San Diego, CA). The libraries were sequenced on the Illumina NovaSeq 6000 platform using 150-bp paired-end reads. Reads were mapped to the human genome (hg38) using a Dragen mapper (v.1.3.0), and a Haplotypecaller (GATK 4.3.0.0) was used to identify SNVs and short indels [8]. Variants were annotated using ANNOVAR-based VARaft (v.2.1.2) and VEP-based seqr (v1.0-x) [9,10]. To identify SVs and large indels, ClinSV using CNVnator and lumpy software was employed [11]. Mobile element insertion was assessed using the Mobile Element Locator Tool (MELT) algorithm [12]. DNA samples from other available family members were screened using Sanger sequencing (BigDye v3.1) for the presence of candidate variants identified using GS.

Variant filtering and annotation: The genomic regions associated with NCMD, encompassing *PRDM13*, *IRX1*, and its upstream regions, were selected. Subsequently, SNVs and small indels were filtered based on the gnomAD v.3.1.2 minor allele frequency (0.5%) and zygosity (heterozygous). The filtered variants were re-assessed using the NHLBI TransOmics for Precision Medicine variant database (TOPMed, freeze 8); the Korean Reference Genome Database (KRGDB), which includes genome sequencing data from 1,722 Koreans [13]; and GEM-J WGA, which includes whole-genome sequencing data from 7,609 Japanese, to identify the population-specific minor allele frequency of population-specific variants [14]. The SVs in these genomic regions were evaluated using the Database of Genomic Variants (DGV). Other coding variants of 290 known retinal disease–associated genes were assessed to identify likely pathogenic or pathogenic variants. The GS workflow is presented in Appendix 2. Candidate variants were investigated for their potential effects on gene regulation and cross-species conservation using the UCSC Genome Browser (Santa Cruz University, Santa Cruz, CA). To identify the epigenetic signature, publicly available ATAC-seq in human and retinal organoids was also analyzed [15].

RESULTS

Clinical phenotypes of affected patients: Six individuals (four from Family A and three from Family B) were examined, and the genomes of the two probands were sequenced using GS. Although data on Family A were reported [13], mutational analysis was not conducted at the time. Fundus photography of the affected family members revealed wide inter-individual variations in grades 1–3 of NCMD lesions with or without subretinal fibrosis. Twenty years after the first report, the proband of Family A (Patient II-5, 35-year-old female) had a best-corrected visual acuity (BCVA) of 20/25 and 20/200 in the right and left eyes, respectively. Fundus photography revealed drusenoid deposits with small retinal excavation and retinal pigment epithelium (RPE) disruption at the fovea in the right eye and large confluent drusen and disciform scars in the macula of the left eye (Figure 1A–E). The clinical features were typical of Grade 2 NCMD in both eyes. An examination of the anterior segment revealed no significant findings.

In Family B, Patient II-5 was a male who was 16 years old at the time of diagnosis. His BCVA was 20/20 in both eyes. A fundus examination revealed multiple drusen-like deposits on the maculae of both eyes. OCT displayed disrupted outer and inner segmented photoreceptor layers and small deposits in the ellipsoid zone on the fovea despite an intact RPE layer on the fovea, which was consistent with Grade 1 NCMD in both eyes (Figure 1F–J). Fundus autofluorescence (AF) revealed multiple speckled hyperAF lesions at the macula in both eyes that reflected the disruption of the photoreceptor layer on OCT. No definite drusen-like lesions were observed in the

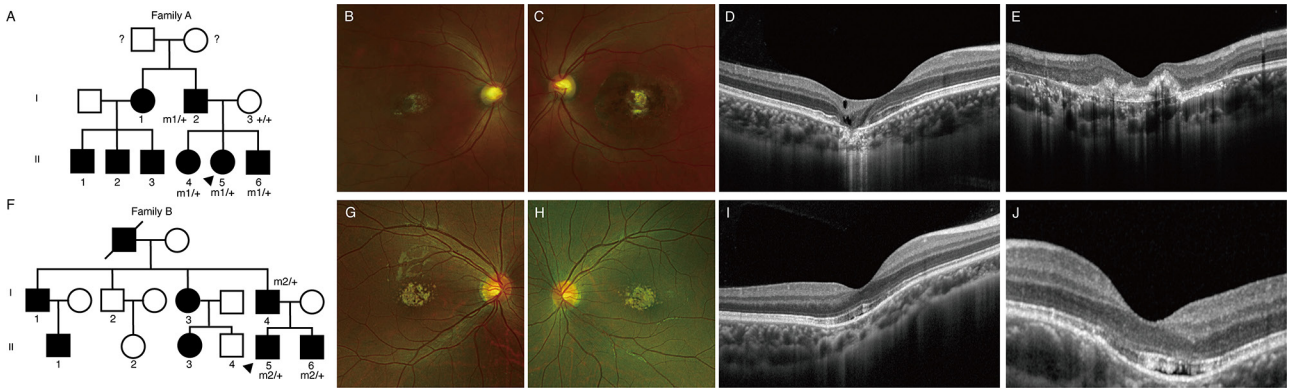

Figure 1. Pedigrees and clinical features in patients with North Carolina macular dystrophy. **A**: Pedigree of Family A. **B**–**E**: Fundus photograph and optical coherence tomography (OCT) of the 35-year-old female (Family A, Patient II-5). Mild retinal excavation with ellipsoid and retinal pigment epithelium defects was noted in the right eye. Larger elevated confluent drusen and disciform scars with pigment clumping were noted in the left eye. **F**: Pedigree of Family B. **G**–**J**: Fundus photograph and OCT of the 16-year-old male (Family B, Patient II–5). Multiple drusen-like deposits on the maculae of both eyes denote Grade 1 North Carolina macular dystrophy (**I**, **J**).

peripheral retina (Appendix 3). The 10–2 central visual field test illustrated no definite scotoma in both eyes.

Patient II-6, the younger brother of Patient II-5, was a 14-year-old boy. His BCVA was 20/20 in both eyes, and a fundus examination revealed multiple small drusenoid deposits in the fovea, similar to those in Patient II-5. OCT revealed a disrupted photoreceptor layer in the right eye and mild RPE layer disruption in the left eye. No definite drusen-like lesions were observed in the peripheral retina (Appendix 4). The central visual field remained intact.

Patient I-4 was a 50-year-old male with a phenotype similar to that of his sons (Appendix 5). Relatively more drusen were observed on the peripheral retina, revealing hyperautofluorescent dot lesions (Appendix 5). His visual acuity was 20/20 in both eyes. Additionally, no definite visual field defect was noted on the 10–2 visual field test (Table 1).

Identification of causative variant in genome sequencing: GS helped identify two rare novel SNVs upstream of *PRDM13* in these two families. Moreover, no copy number variations, structural variants, or mobile element insertions were detected in the neighboring *PRDM13* or *IRX* genes. No rare pathogenic coding variants of the known inherited retinal dystrophy genes exist. These two novel variants, named V17 and V18, were in NCMD mutational hotspot 2 (*PRDM13_cis*-regulatory elements; Figure 2A), which was reported as an enhancer interacting with the promoter of *PRDM13* [16]. The publicly available single nucleus ATAC analysis revealed that this region is enriched in early retinal progenitor cells of both the human retina and organoid (Appendix 6) [15].

The chr6:g.99,598,914T>C (V17) variant was identified in Patient II-5 (Family A) after filtering the common variant (MAF <0.5%). However, it was not present in gnomAD v.3.1.2 and TOPMed databases. This variant was also absent from the East Asian genomic databases KRGDB or TOGOVAR. V17 was 7 bp downstream of the previously reported V10 variant. Segregation analysis identified this variant in three other affected individuals (patients I-2, II-4, and II-6). This variant is not present in the unaffected mother (Family A, I-3, Figure 1A). In Family B, chr6:g.99,598,926G>A (V18) was identified in Patient II-5, and this variant was not present in population genetic databases such as gnomAD, TOPMed, KRGDB, and TogoVar. Segregation analysis revealed that this variant was present in two other affected family members (Family B, patients I-4 and II-6, Figure 1F).

We used the FABIAN variant to predict the effect of DNA variants on transcription factor binding [17]. This analysis suggested that the V17 variant causes the loss of the transcription factor binding, such as POU2F1–3 and POU3F1–2, belonging to the Pit-Oct-Unc (POU) family. The V18 variant disrupts the binding of the SIX2 transcription factor (Appendix 7 and Appendix 8).

Clinical interpretation of the pathogenicity of variants: We assessed the pathogenicity of the variants using guidelines recommended for non-coding regions [18]. We obtained evidence supporting the pathogenicity of both variants. These two variants were in mutational hotspots without benign variations (PM1) and were absent from the population databases (PM2). The variant is co-segregated with multiple affected family members in a gene known to cause the disease (PP1). The phenotype of a patient is highly specific to monogenic diseases (PP4). According to these guidelines, these two putative candidate variants were classified as likely pathogenic.

Table 1. Clinical phenotype of affected patients in two unrelated families with North Carolina macular dystrophy.

Patient No.	Sex /Age	Refraction (SE)		BCVA		Funduscopic findings	OCT findings
		OD	OS	OD	OS		
I-2 (Family A)	M/44	+0.38	+0.63	20/20	20/20	Confluent drusen at parafovea (OD) Fine macular drusen-like deposits (OS)	Focal disruption of ellipsoid zone without retinal excavation (OU)
II-4 (Family A)	F/18	−0.50	−0.88	20/100	20/200	Yellowish fibrotic scars with surrounding geographic atrophy in the macula (OU)	Intraretinal cavitation and subretinal fibrosis with retinal excavation (OU)
II-5 (Family A)	F/35	−1.63	−1.00	20/25	20/200	Confluent drusen, mild RPE atrophy and excavation in the macula (OD) Yellowish fibrotic scars with surrounding geographic atrophy in the macula (OS)	Small retinal excavation with disruption of ellipsoid zone (OD) Elevated confluent drusen, subretinal fibrosis, and disciform scars without retinal excavation (OS)
II-6 (Family A)	M/12	0.00	+0.13	20/40	20/20	Subretinal fibrosis and pigment clumping (OU)	Focal defect of RPE and photoreceptor layers (OU)
I-4 (Family B)	M/54	−2.50	−2.50	20/20	20/20	Confluent drusen at fovea (OU)	No retinal excavation with foveal vitelliform lesion (OD) A little retinal excavation with deposits in ellipsoid zone (OS)
II-5 (Family B)	M/17	−4.00	−3.75	20/20	20/20	Confluent drusen at fovea (OU)	Small retinal excavation with disruption and deposits in ellipsoid zone (OU)
II-6 (Family B)	M/14	−0.50	−0.25	20/20	20/20	Confluent drusen at fovea (OU)	No retinal excavation with disruption of ellipsoid zone (OU)

F=female; M=male; OD=right eye, OS=left eye, OU=both eyes, SE=spherical equivalent, BCVA=best corrected visual acuity, OCT=optical coherence tomograph.

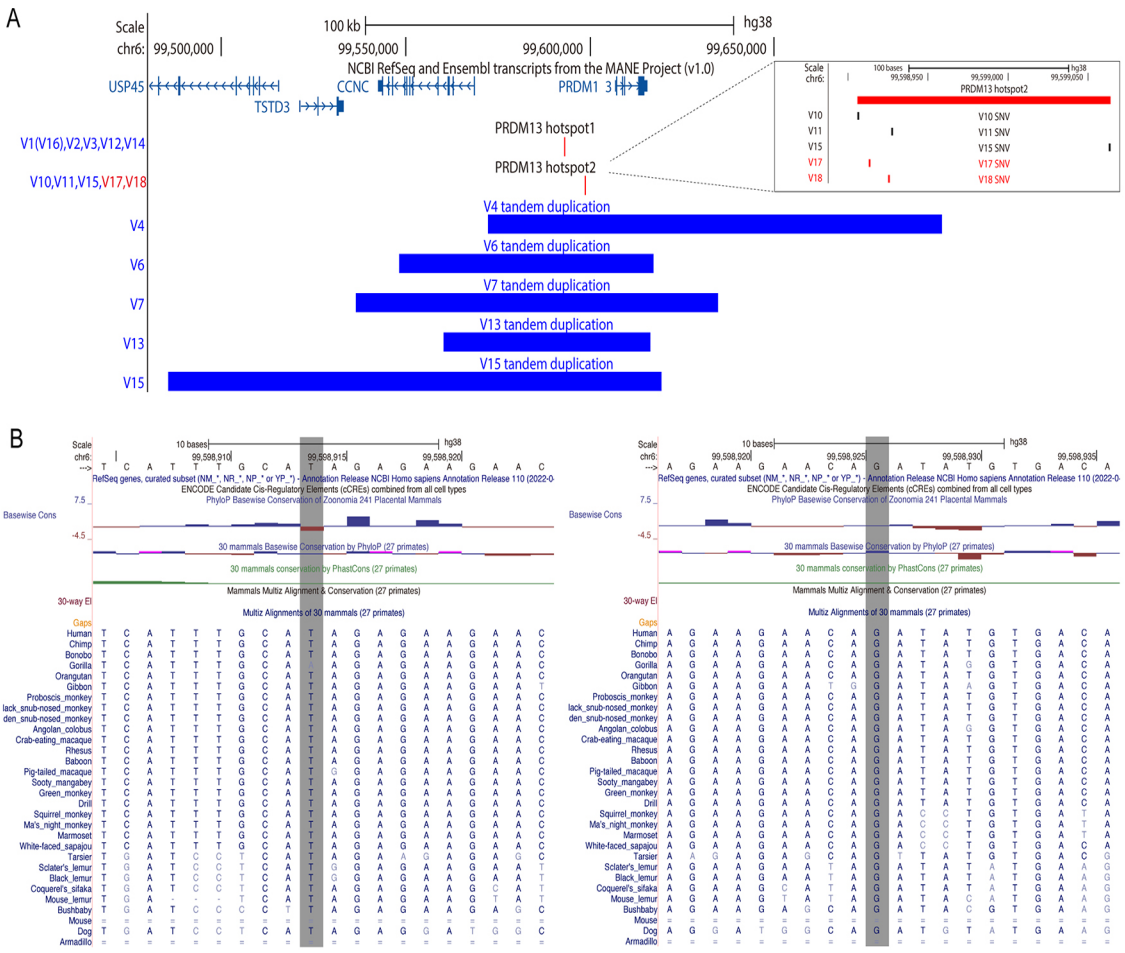

Figure 2. Previously reported variants and conservation map of novel variants in *PRDM13*. **A**: Schematic of the two identified novel variants associated with North Carolina macular dystrophy at the 6q15 locus in the UCSC genome browser. V17 (GRCh38/hg38 chr6:99,598,914T>C in Family A) and V18 (GRCh38/hg38 chr6:99,598,926G>A) were located between V10 and V11 (mutational hotspot-2). **B**: Conservation map for V17 and V18 in 28 mammals. Both variants are well conserved within primates and mammals.

DISCUSSION

In this study, we identified two novel non-coding SNVs in two unrelated East Asian families with NCMD. These novel variants were absent from the population genetic database. These variants were located within a previously identified mutational hotspot-2 (PRDM13_cCRE5). Publicly available ATAC-seq, DNA-seq, and methylation studies also indicated that these variants are in the cis-regulatory element [16]. Furthermore, the nucleotides of these positions are highly conserved in 28 mammalian species. Notably, despite the proximity of the two variants, the severity of the disease of the two variants differed substantially. Variant V17 causes Grade 2 NCMD with some intra-eye and intra-familial variability, whereas variant V18 causes Grade 1 NCMD with normal visual acuity. Collectively, our findings suggest that these novel variants are associated with PRDM13 dysregulation.

The newly discovered novel variants, V17 and V18, were between V10 and V11 on mutational hotspot 2. Markedly, these variants are in DNase I hypersensitive sites (DHS), which are open and active at the developmental stage of the retina, primarily in the early developmental stages of retinal progenitor cells. Green et al. used the activity-by-contact (ABC) model to predict the effects of enhancers on the regulation of target genes using epigenomic data from human embryonic stem cells [19]. V10 was identified as functional in the development of both macular and retinal tissues. V17 and V18 fall within the same enhancer region. Hence, they may affect the development of macular and retinal tissues.

During human embryonic development, mutational hotspot-2 (PRDM13_cCRE5) was identified as an active cCRE on Day 103, when the retinal progenitor cells of the macula stop mitosis and differentiate to their photoreceptor fate [16]. Variants in PRDM13 regulatory regions could mainly affect macular development. The dysregulation of PRDM13, and possibly overexpression, likely leads to developmental macular dystrophy. Recently, biallelic loss-of-function *PRDM13* variants have been reported to cause fatal perinatal brainstem dysfunction with cerebellar hypoplasia [20,21]. Further studies are required to elucidate the role of PRDM13 in foveal development.

Moreover, mutational hotspot-2 could be a crucial region for determining the spectrum of phenotypes. Variants in mutational hotspot-1 revealed typical NCMD, whereas variants in mutational hotspot-2 demonstrated wide phenotypic variability among NCMD, progressive bifocal chorioretinal atrophy (PBCRA), and congenital posterior polar chorioretinal hypoplasia (CPPCRH; Appendix 9). The genetic loci causing PBCRA were initially mapped to the region (6q14–16.2) that overlapped with the MCDR1 locus [22]. Individuals affected with PBCRA had poor visual acuity in early infancy, nystagmus, and myopia with slowly progressive atrophic macular lesions. In patients with PBCRA, an electroretinogram (ERG) revealed generalized rod and cone photoreceptor dysfunction [23,24]. Typical patients with NCMD exhibit relatively better visual acuity during their lifetimes than those with PBCRA. However, the two diseases share similar phenotypic features in the fundus findings, suggesting a common underlying etiology. Subsequently, NCMD and PBCRA have the same genetic causalities in the non-coding regions of *PRDM13*, which leads to the dysregulation of PRDM13 expression [25]. The V1–V3, V12, and V16 in mutational hotspot-1 and tandem duplications encompassing *PRDM13* presented with typical NCMD, not PBCRA [5,26]. In mutational hotspot-2, V11 indicated PBCRA characterized by the slow progression of atrophic lesions at the macula in early life [25]. Individuals affected with V10 exhibited typical features of NCMD, although 3-year-old child with V10 variant presented more severe macular atrophy with the progression of the macular lesion [25]. This was defined as an autosomal dominant disease in 11 affected patients of a single Egyptian family and was renamed CPPCRH by Small et al. [27]. V15 revealed typical NCMD without severe visual impairment [28]. The affected individuals in our study (V17, V18) demonstrated clinical features closer to typical NCMD. This phenotypic variability also suggests that variants in mutational hotspot-2 contribute to either NCMD or PBCRA.

The interpretation of non-coding variants is challenging because they act via variable mechanisms. The variants in non-coding regions may regulate gene expression by altering transcription factor binding, creating new topologically associated domains, generating or disrupting upstream open reading frames, or forming strong G-quadruplex structures to prevent transcription initiation. Furthermore, they often have gene-specific effects depending on the spatiotemporal activities of other cCREs. For example, the zinc finger protein CCCTC-binding factor (CTCF) plays a role in the regulation of genome and chromatin structures. However, disrupting the CTCF-associated domain demonstrated significant pathogenicity, resulting in limb malformation [29], whereas it did not in cancer cells [30]. Therefore, guidelines for interpreting non-coding variants recommend that variants in candidate CREs should be validated using functional experiments demonstrating the direct effect of the CRE in relevant tissues on appropriate developmental stages. Although we could not perform functional validation, the variants were on PRDM13_cCRE5, an established causative variant affecting *PRDM13* expression [16]. In-vivo enhancer assays in *Xenopus* displayed eye- and brain-specific activity for PRDM13_cCRE5 during embryogenic development. Therefore, we speculated that these novel variants likely act as possible pathogenic variants of NCMD.

The analysis with a FABIAN variant revealed that transcription factor-binding motifs (POU family and SIX2), which are essential for retinal development, were altered [31,32]. This corroborates previous results [15,16,19]. However, the alteration of transcription factor-binding motifs differed between variants in NCMD; therefore, no single transcription factor was attributed to NCMD pathogenesis. Therefore, the precise mechanisms by which these genomic regions regulate gene expression warrant further investigation.

Our study has some limitations. We could not validate variants using well-established functional experiments. However, we analyzed the variants using FABIAN-variant and ATAC-seq data, which supported the potential pathogenicity of these variants. Second, we could not determine the precise epigenetic mechanism underlying V17. Despite the detection of the same variant in the four family members, there was phenotypic variability. Therefore, further studies are needed to investigate the mechanism responsible for the variable expressivity in NCMD. Despite these limitations, our robust bioinformatics analysis excluded other possible causative variants in families with NCMD. Last, although progression is rare in typical NCMD or considerably slow in PBCRA, the long-term analysis of retinal structural changes and visual acuity was lacking in our study.

Recently, an increasing number of coding and non-coding SNVs resulting in inherited retinal diseases have been identified [33–36]. Most reported non-coding SNVs affect aberrant splicing, whereas only a few non-coding cis-regulatory variants have been identified in inherited retinal diseases (IRD) [34,35]. NCMD can be considered a model for investigating non-coding regulatory variants in IRD. Further studies of patient-derived cells may help elucidate the underlying mechanisms of this *cis*-regulatory disease. Additionally, the identification of these *cis*-regulatory variants raises the need to extend the areas captured by targeted panels by including known disease-causing pathogenic regulatory promoters, enhancers, or non-coding exons in addition to coding exons.

APPENDIX 1.

To access the data, click or select the words "Appendix 1." Reported and novel variants in PRDM13 associated with North Carolina macular dystrophy.

APPENDIX 2.

To access the data, click or select the words "Appendix 2." Genome sequencing analysis workflow.

APPENDIX 3.

To access the data, click or select the words "Appendix 3." Clinical findings of subject II-5.

APPENDIX 4.

To access the data, click or select the words "Appendix 4." Clinical findings of subject II-6.

APPENDIX 5.

To access the data, click or select the words "Appendix 5." Clinical findings of subject 1-4.

APPENDIX 6.

To access the data, click or select the words "Appendix 6." Single nucleus characterization of chromatin accessibility in developing and adult retina and human retinal organoids.

APPENDIX 7.

To access the data, click or select the words "Appendix 7." Transcription factor binding site (TFBS) gain and loss due the variant V17 (chr6:g.99,598,914T>C).

APPENDIX 8.

To access the data, click or select the words "Appendix 8." Transcription factor binding site (TFBS) gain and loss due the variant V18 (chr6:g.99,598,926G>A).

APPENDIX 9.

To access the data, click or select the words "Appendix 9." Clinical feature and region of reported and novel PRDM13 small nucleotide variants in patients with North Carolina macular dystrophy.

ACKNOWLEDGMENTS

Dr. Jinu Han (jinuhan@yuhs.ac) and Dr. Se Joon Woo (sejoon1@snu.ac.kr) are co-corresponding authors for this study. This study was presented as abstract in 2023 ARVO Annual Meeting, held in New Orleans, LA, April 23–27, 2023. Funding: This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (the Ministry of Science and ICT; No.2020R1C1C1007965 and No.2022R1A2C4002114). Disclosures: Y. Seo, None; K. Joo, None; J Lee, None; A. Diaz, None; S. Jang, None; T.J. Cherry, None; K.M. Bujakowska, None; J. Han, None; S.J. Woo, None; K.W. Small, None. Data and code availability: All the sequencing data supporting the findings of this study are readily available from the corresponding author upon request. The codes for genome sequencing analysis using DRAGEN-GATK are available from GitHub (https://github.com/jin0008/Dragen-GATK). Pathogenic variant data were submitted to the Leiden Open Variation Database (LOVD V17: 00,433,782; V18: 00,433,866). Genome sequencing data are not publicly available as they can compromise research participant privacy.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 19 February 2024. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.