Decreased Levels of DNA Methylation in the *PCDHA* Gene Cluster as a Risk Factor for Early-Onset High Myopia in Young Children

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Purpose. High myopia (HM), an eye disorder with at least –6.0 diopters refractive error, has a complex etiology with environmental, genetic, and likely epigenetic factors involved. To complement the DNA methylation assessment in children with HM, we analyzed genes that had significantly lower DNA methylation levels.

Methods. The DNA methylation pattern was studied based on the genome-wide methylation data of 18 Polish children with HM paired with 18 controls. Genes overlapping CG dinucleotides with decreased methylation level in HM cases were assessed by enrichment analyses. From those, genes with CG dinucleotides in promoter regions were further evaluated based on exome sequencing (ES) data of 16 patients with HM from unrelated Polish families, Sanger sequencing data of the studied children, and the RNA sequencing data of human retinal ARPE-19 cells.

Results. The CG dinucleotide with the most decreased methylation level in cases was identified in a promoter region of *PCDHA10* that overlaps intronic regions of *PCDHA1–9* of the *PCDHA* gene cluster in myopia 5q31 locus. Also, two single nucleotide variants, rs200661444, detected in our ES, and rs246073, previously found as associated with a refractive error in a genome-wide association study, were revealed within this gene cluster. Additionally, genes previously linked to ocular phenotypes, myopia-related traits, or loci, including *ADAM20*, *ZFAND6*, *ETS1*, *ABHD13*, *SBSPON*, *SORBS2*, *LMOD3*, *ATXN1*, and *FARP2*, were found to have decreased methylation.

Conclusions. Alterations in the methylation pattern of specific CG dinucleotides may be associated with early-onset HM, so this could be used to develop noninvasive biomarkers of HM in children and adolescents.

Keywords: hypomethylation, epigenetic modifications, myopia candidate genes, *PCDHA10*, protocadherin alpha

High myopia (HM), defined as an eye disorder with a refractive error (RE) of –6.0 diopters (D) or higher, is a major cause of blindness in developed countries.[1,2] Near work,[3] artificial light exposure,[4] lack of physical activity outdoors,[3,5,6] a higher level of education[7] and urbanization,[8–10] or a diet with high sugar intake[11] are main environmental factors for HM worldwide. Near work, computer work, reading, and writing require intensive eye accommodation,[12–14] and children who spend more time on said activities have increased myopic RE.[13,14] The eyeballs of children who mainly spend time at home in artificial light were longer and their risks of myopia were higher than those who primarily spent time outdoors in natural light.[4,12,15,16] In Poland, myopia is more prevalent among children living in urban areas than in the countryside.[10]

Thus far, 26 myopia loci have been documented in the Online Mendelian Inheritance in Man (OMIM), including 13 HM loci. In addition, a significant number of population- or family-specific candidate genes and sequence variants have been revealed, as reviewed in Cai et al.[17] In 2011, we identified HM loci at 7p22.1–7p21.1, 7p12.3–7p11.2, and 12p12.3–12p12.1,[18] in which several genes were associated with HM (*AGMO*),[19] myopia/RE (*COBL*, *C1GALT1*, *THSD7A*, *AHR*, *PDE3A*, *ETNK1*, *ST8SIA1*, *C2CD5*, *OR7E136P*, and *UNC93B2*),[19–22] or astigmatism (*EGFR* and *ABCA13*)[23,24] in genome-wide association studies (GWASs). We did not confirm association of the *IGF1* gene with myopia phenotypes,[25] but we have recently found variants in *FLRT3* and *SLC35E2B* genes, segregating with the HM phenotype in Polish patients.[26] In addition to genetic and

environmental factors discussed as causative in HM development,[27–30] a few reports have been published on DNA methylation in myopia.[31–35] However, these results are inconsistent and need to be verified across different/larger populations. As our previously published study focused on increased methylation level of CG dinucleotides in HM,[36] here, to complete the earlier assessed aspects of hypermethylation, we performed additional analyses to point to the genes with decreased levels of methylation. That allowed us to evaluate the role of hypomethylation in HM and therefore complement the previous findings on HM in young children.

Materials and Methods

Patients

A total of 27 Caucasian Polish children under the age of 12 years with HM and 24 children without HM were ascertained in the Department of Ophthalmology and Eye Rehabilitation at the Medical University of Bialystok. Details of the study cohort have been previously published.[36] Briefly, all the children underwent an eye examination, including cycloplegic (cyclopentolate 1%) autorefraction, and ocular biometry measurements. The guidelines of the International Myopia Institute that define HM as a spherical equivalent RE ≤ -6.00 D when ocular accommodation is relaxed[2] were followed. The ascertained children presented with a minimum RE of –6.0 D in at least one eye and a minimum axial length of 26 mm. The control group consisted of children without HM, with axial length below 26 mm. No genetic diseases were diagnosed in the studied children. The study protocol was approved by the Institutional Review Boards at Poznan University of Medical Sciences in Poland, and written informed consent in accordance with the Declaration of Helsinki was obtained from the parents of each minor participant after explanation of the nature and possible consequences of the study.

Bioinformatic and Statistical Analyses of Genome-Wide DNA Methylation Results

Genome-wide methylation analyses were previously performed on genomic DNA samples extracted from peripheral blood of 18 Polish children with HM and 18 age-matched controls using Infinium MethylationEPIC BeadChip arrays (Illumina, Inc., San Diego, CA, USA) covering 850,000 methylation sites.[36] The detailed methodology was described elsewhere.[36]

In this study, genome-wide DNA methylation data were analyzed to select CG dinucleotides with a significantly lower methylation level in HM. Mean methylation values were calculated for the group of children with HM and the group of children without HM. To avoid any gender-specific methylation bias, CG dinucleotides located on chromosomes X and Y were excluded from the analysis. Strict selection criteria were employed for the CG dinucleotides: (1) a minimum 15% difference in the mean methylation levels between HM cases and controls, (2) localization in gene or promoter region, (3) false discovery rate (FDR)–corrected P value <0.00001, and (4) no overlap with single-nucleotide polymorphisms (SNPs) to avoid potential confounding factors. An overview of the study workflow is presented in Figure 1.

Based on the set of preselected differentially methylated CG dinucleotides, those with at least a 20% methylation decrease and located within the 0 to 200 bases upstream of the transcriptional start site (TSS200), 5′ untranslated region (UTR), exon 1, or 200 to 1500 bases upstream of the transcriptional start site (TSS1500) were chosen to identify genes with possibly altered expression due to differential methylation of the promoter region (Fig. 1). We considered them as the highest-ranked CG dinucleotides.

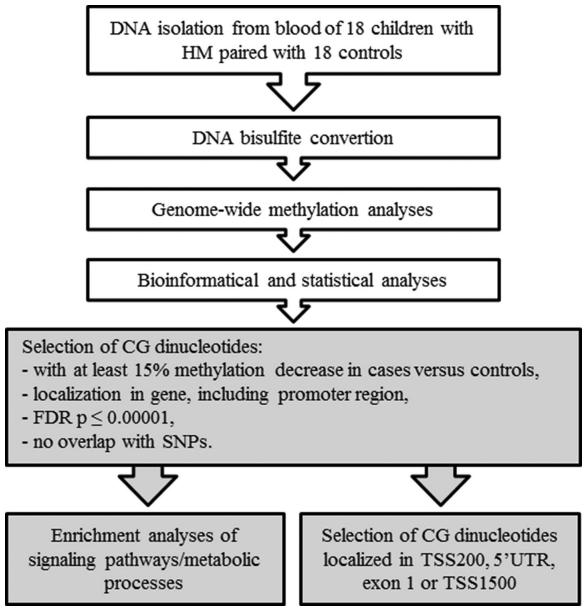

Figure 1. Detailed workflow of DNA methylation analyses in children with HM and controls. The research steps marked in *white* have been already published.[36] Boxes colored in *gray* indicate the steps performed in the current study.

Characteristics of genes overlapping the highest-ranked CG dinucleotides, including expression and function, were assessed in GeneCards (https://www.genecards.org/),[37] National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/),[38] UniProt (https://www.uniprot.org/),[39] Genome Browser (http://genome.ucsc.edu),[40] GWAS Catalog (https://www.ebi.ac.uk/gwas/),[41] Mouse Genome Informatics (MGI; http://www.informatics.jax.org/),[42] and available literature data. First, genes with reported function or association with ocular tissue/eye disorder and/or localized at the myopia locus were assessed. Next, genes with confirmed expression in ocular tissue but unknown function in the eye were compiled and analyzed. Finally, genes that are neither expressed in the eye nor associated with myopia/eye were examined.

We investigated the possible transcription factor binding sites of single nucleotide variants (SNVs) in chosen genes using PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3)[43,44] and Genome Browser.

Methylation, RNA Sequencing, and Exome Sequencing Data Correlations

The expression profiles of differentially methylated genes were retrieved from publicly available raw RNA sequencing data of human retinal pigment epithelial (RPE) cells (ARPE-19; GEO: GSE88848).[45] Although no particular cell line appears to be viable for myopia research, RPE cells are involved in its development as their density changes with

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axial elongation.[46,47] Transcript levels expressed in transcripts per million (TPM) units and measured at 4 days and 4 months of culture were analyzed.

To investigate the connection between genetic and epigenetic features in HM, the methylation data were combined with previously obtained exome sequencing (ES) results for 16 patients with HM selected from seven unrelated Polish families with HM.[26]

Sanger sequencing was performed for genotyping of selected sequence variants in the studied group of Polish children and segregation analyses in a Polish family.

Enrichment Analyses of Genes Overlapping the CG Dinucleotides With Decreased Methylation

Genes with CG dinucleotides with at least a 20% methylation difference were evaluated by enrichment analyses in Gene Ontology (GO; http://geneontology.org/)[48–50] and the GO enrichment analysis and visualization tool (GOrilla; http://cbl-gorilla.cs.technion.ac.il/).[51,52] Pathways/processes with a q value <0.01 were considered significant. The same gene list was also applied in overrepresentation analyses of signaling pathways and metabolic processes in ConsensusPathDB (http://cpdb.molgen.mpg.de/) (Fig. 1).[53] The pathways that shared at least three genes with our gene set and had a P value adjusted for FDR (q value) <0.01 were considered.

Statistical Analyses

We performed a statistical analysis of genotype distribution using Fisher's exact test. P values <0.05 were considered statistically significant.

Results

Patients' Characteristics

Detailed characteristics of the enrolled patients are presented elsewhere.[36] Briefly, all the children were Caucasians, between the ages of 3 and 12 years. Children with HM presented with an RE ranging from −6.0 to −15.0 D in at least one eye (mean value of –8.25 D) and axial lengths in the range of 26.22 to 27.85 mm (mean value of 26.22 mm) (Supplementary Table S1). Children in the control group had an RE ranging from –0.5 to +0.5 D (mean, –0.25 D) and an axial length ranging from 22.42 to 24.11 mm (mean, 22.55 mm) and no signs of HM. The anterior segment of the eye was normal in all examined children.

Characteristics of CG Dinucleotides With Decreased Methylation Level

Of the 865,918 detected CG dinucleotides, 616,598 were located within a gene or its promoter. Among them, 56 dinucleotides had at least a 20% (Supplementary Table S2) lower methylation level, comparing HM and control samples. Results of detailed statistical analyses of the highest-ranked CG dinucleotides are presented in Table and Figure 2. The cg27494055 mapped within a TSS1500 region of *PCDHA10* (OMIM:606316) had the greatest difference in methylation level between patients with HM and controls. As this TSS1500 region and the *PCDHA10* gene sequence overlap with other genes from a protocadherin α (*PCDHA*) gene cluster, the cg27494055 is also localized in intronic regions of *PCDHA* genes 1 to 9 (Fig. 3).

In Supplementary Table S3, we list genes associated with eye phenotypes and myopia risk factors identified in GWASs and/or ocular phenotypes caused by gene mutations in mice. The MGI database shows that most of the indicated genes are expressed in a murine retina (GXD: E-GEOD-63810, GXD: E-GEOD-33141, GXD: E-MTAB-6133) (Supplementary Table S3).

Expression of the Assessed Genes in the ARPE-19 Cell Line

The expression data, presented in TPM, for genes with the highest-ranked CG dinucleotides were slightly different in the two time points (4 days and 4 months) of culture of the ARPE-19 cell line. Whereas expression of *ZFAND6* (OMIM:610183), *ETS1* (OMIM:164720), *ABHD13*, *LIG4* (OMIM:601837), and *TANC1* (OMIM:611397) was detected in both culture time points, expression of the *PAG1* (OMIM:605767) and *ATXN1* (OMIM:601556) genes was observed only after 4 months of the ARPE-19 culture. The highest expression levels were reached by the *ZFAND6* gene, after 4 days (98.20 TPM) and 4 months of the culture (89.91 TPM). Detailed expression data for selected genes were compiled in Supplementary Table S4.

Methylation and Genomic Data Correlations

A comparison on methylation data and previously obtained genomic ES data from Polish patients with familial HM[26] was conducted. Genes that overlap the highest-ranked CG dinucleotides (Table) were examined for variants using the previously obtained ES data. As a result, four sequence variants within the coding sequence of *PCDHA10*, *ABHD13*, and *ATXN1* were detected. The SNP rs150882242, detected in *ABHD13*, was predicted to be deleterious by SIFT and MutationTaster and possibly damaging by Polyphen2. Two variants were identified in *ATXN1*, but both were predicted as benign.

A nonsynonymous SNV, rs200661444 (c.2017C>T, p.(Q673X)) (Fig. 3) in *PCDHA10*, detected in our ES, is a nonsense variant that infers a high risk of deleterious effect according to MutationTaster. According to JASPAR, the SNV overlaps with the binding sites of transcription factors NFIX and Zfx. The rs200661444 was detected in patients with HM from family HM-78, but it was not detected in any of the 18 studied HM children (Supplementary Table S5). We present the HM-78 pedigree with the results of the allele segregation analyses in Figure 4. Also, we found a disturbance in segregation of the rs200661444 in the HM-78 family in HM-78-07, HM-78-10, HM-78-11, and HM-78-08, which is caused by a 16,794-bp deletion, starting in exon 1 of *PCDHA8* and ending in intron 1 of *PCDHA10*. The studied one HM (UR-819) and four control children (UR-840, UR-847, UR-851, UR-852) were the carriers of the deletion. Checking the data of individual methylation values obtained for the evaluated samples, we found that the deletion itself did not influence the level of methylation of the cg27494055 in the listed children.

The SNV rs1581364290, overlapping cg27494055, was neither detected in samples from members of the HM-78 family (Fig. 4) nor in the 18 HM or control children tested. We checked also sequence variant rs246073 in *PCDHA10* in our patients, and it was detected in the HM-78 family but did not completely segregate with the HM phenotype

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Table. Highest-Ranked CG Dinucleotides Located in Gene Promoter Regions With at Least 20% Decrease in the Methylation Level in HM Cases When Compared to Controls

Target ID	Gene	Chromosomal Localization	*P* Value	FDR *P* Value	Methylation Level in HM Cases, Mean ± SD (Range), %*	Methylation Level in Controls, Mean ± SD (Range), %*	Difference in Methylation Level	Localization in Gene	Distance to CpG Island
A. Genes related to myopia, eye structure, or function									
cg27494055	*PCDHA10*	5q31.3	5.26×10^{-28}	4.47×10^{-22}	29.63 ± 27.2 (2.1–86.2)	56.70 ± 28.9 (3.8–86.7)	−27.07	TSS1500	N Shore
cg09701422	*ADAM20*	14q24.2	7.47×10^{-28}	6.35×10^{-22}	63.66 ± 26.0 (18.4–90.1)	89.87 ± 2.4 (83.6–93.2)	−26.21	TSS1500	
cg24017056	*ZFAND6*	15q25.1	1.72×10^{-28}	1.46×10^{-22}	59.63 ± 23.4 (7.8–88.3)	83.28 ± 4.6 (72.0–90.1)	−23.64	5′ UTR	
cg27078890	*ETS1*	11q24.3	1.06×10^{-28}	9.04×10^{-23}	65.25 ± 23.6 (7.7–89.0)	88.02 ± 2.7 (83.1–92.1)	−22.76	TSS200	
cg15039162	*ABHD13*	13q33.3	9.49×10^{-29}	8.07×10^{-23}	62.27 ± 25.1 (13.8–88.0)	84.72 ± 3.4 (78.8–89.6)	−22.46	5′ UTR	S Shore
cg19518093	*SBSPON*	8q21.11	8.99×10^{-29}	7.64×10^{-23}	67.98 ± 26.0 (7.2–91.6)	90.18 ± 1.8 (87.0–93.4)	−22.20	TSS1500	S Shore
cg27262015	*SORBS2*	4q35.1	6.53×10^{-29}	5.55×10^{-23}	64.35 ± 26.1 (6.2–90.5)	86.16 ± 3.5 (79.3–91.4)	−21.82	TSS200	
cg12836825	*LMOD3*	3p14.1	4.92×10^{-29}	4.18×10^{-23}	66.22 ± 21.5 (5.0–90.0)	87.39 ± 4.9 (72.5–94.6)	−21.16	TSS1500	
cg26393261	*ATXN1*	6p22.3	3.82×10^{-29}	3.24×10^{-23}	43.33 ± 18.3 (2.0–64.3)	64.38 ± 4.7 (57.0–74.8)	−21.05	5′ UTR	
cg24684709	*FARP2*	2q37.3	7.03×10^{-25}	5.97×10^{-19}	45.02 ± 21.7 (1.2–73.1)	65.44 ± 11.1 (39.8–76.3)	−20.42	5′ UTR	S Shelf
B. Genes expressed in the eye but with unknown association with myopia/eye									
cg25010006	*PAG1*	8q21.13	2.42×10^{-28}	2.06×10^{-22}	62.06 ± 22.5 (13.9–86.5)	86.48 ± 2.6 (82.6–93.3)	−24.43	TSS1500	S Shore
cg15039162	*LIG4*	13q33.3	9.49×10^{-29}	8.07×10^{-23}	62.27 ± 25.1 (13.8–88.0)	84.72 ± 3.4 (78.8–89.6)	−22.46	TSS1500	S Shore
cg18556587	*TANC1*	2q24.2	4.05×10^{-29}	3.44×10^{-23}	53.42 ± 20.2 (3.8–76.8)	74.74 ± 4.9 (64.3–81.4)	−21.32	5′ UTR	
C. Genes not expressed in the eye and not associated with myopia/eye									
cg11683966	*SLC25A3P1*	1p32.3	4.41×10^{-29}	3.75×10^{-23}	49.46 ± 23.6 (4.0–76.8)	70.84 ± 3.8 (64.8–78.3)	−21.38	TSS200	S Shore
cg05740739	*OR6B3*	2q37.3	2.25×10^{-29}	1.92×10^{-23}	55.98 ± 18.8 (4.3–79.2)	76.12 ± 3.7 (70.6–83.0)	−20.14	TSS1500	

N Shore, region up to 2 kb downstream from CpG island; S Shelf, region up to 2 kb upstream from the S Shore; S Shore, region up to 2 kb upstream from CpG island.

* Methylation levels in children are presented as mean values.

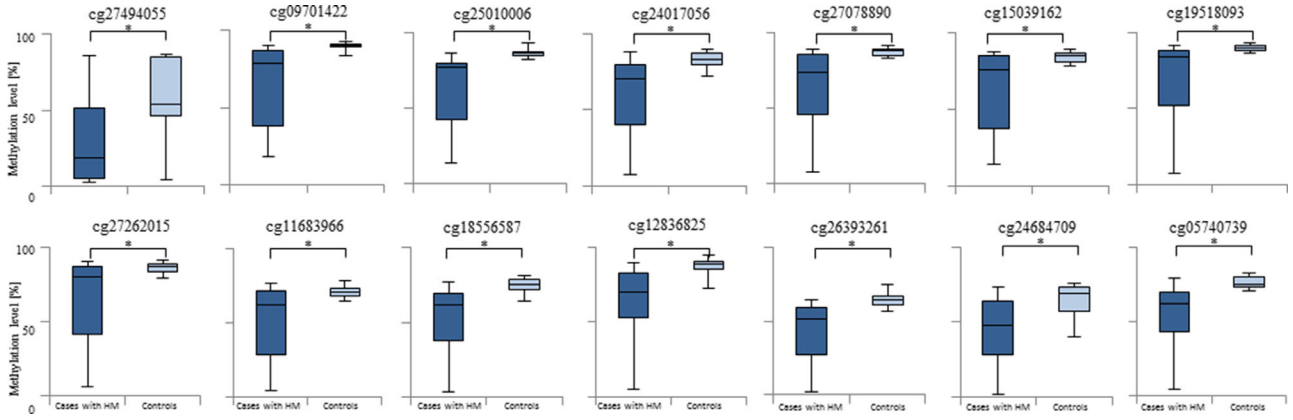

Figure 2. Comparisons of methylation levels of selected CG dinucleotides between cases with HM and controls. Presented are the highest-ranked CG dinucleotides, with at least a 20% methylation difference between HM cases and controls and location in gene promoter regions. Standard deviation is included and *asterisk* (*) stands for the statistically significant difference in methylation level (FDR-corrected $P < 0.01 \times 10^{-16}$)

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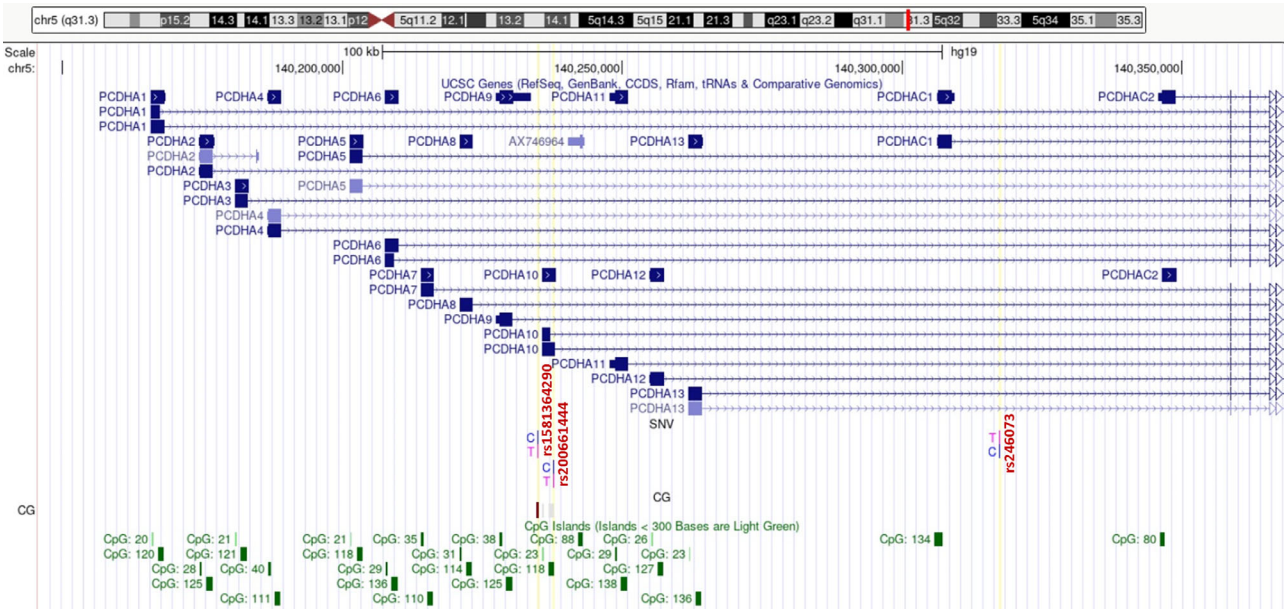

Figure 3. Genes of *PCDHA* cluster overlapping cg27494055 with decreased methylation level in HM children. The *PCDHA* gene cluster is tandemly localized on chromosome 5q31 and it consists of 15 genes and one pseudogene. The 13 upstream genes and the pseudogene have highly similar sequence, while a subfamily C contains two more (C1 and C2) distantly related coding sequences. The CG dinucleotide is localized in TSS1500 of *PCDHA10* gene and in introns of *PCDHA1–9*. SNVs rs200661444 in exon 1 of *PCDHA10*, rs246073, and rs1581364290 colocalized with CG dinucleotide were indicated.

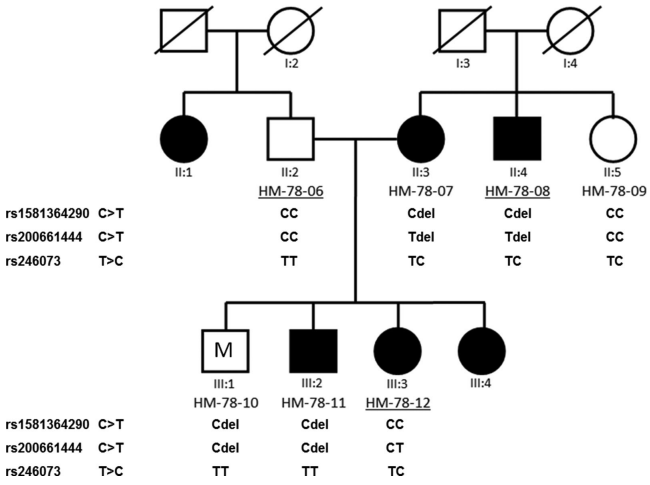

Figure 4. Pedigree of the HM-78 family showing the results of segregation analyses of rs1581364290, rs200661444, and rs246073 in the *PCDHA10* gene. Individuals with HM are indicated *by black-filled symbols* and control individuals by the *open symbols*, whereas symbol with M represents individual (21 years old) with myopia (OD: –4.0 D, axial length (AL) 24.11 mm, OS: –4.5 D, AL 24.82 mm). Individuals assessed in the segregation analysis are numbered under their symbols in the pedigree. Exome sequencing was applied for individuals with the *underlined numbers*. Members HM-78-07, HM-78-10, HM-78-11, and HM-78-08 carried a 16,794-bp deletion, starting in exon 1 of *PCDHA8* and ending in intron 1 of *PCDHA10* (marked by "del").

(Fig. 4). TT, TC, and CC genotypes were detected in both groups, but TC and CC genotypes were present more frequently in the control group (61.1% and 22.2%) than in children with HM (44.4% and 5.6%). No statistically significant difference was found between the distribution of normal and changed genotypes in the studied groups of children ($P = 0.075$).

By Sanger sequencing, we also detected SNV rs251360 G>A in cg27494055. Whereas the wild-type genotype was less frequent in HM cases (5.9%) than in controls (28.6%), genotype GA was common in both groups (47.1% in cases and 57.1% in controls), and AA was more frequent in cases (47.1%) than in controls (14.3%). The distribution of normal and changed genotypes in the studied groups of children was not statistically significant ($P = 0.1484$). In the latter analysis, five children, who carried the identified 16,794-bp deletion, were excluded.

The 5q31 region and other known myopia/HM loci found at chromosomes 5, 7, and 12 are shown in Figure 5. All the CG dinucleotides with at least 15% decreased methylation level in cases versus controls were marked (Fig. 5).

Moreover, the list of 73 previously studied HM putative genes characterized by ES, Sanger, and segregation analyses[26] was assessed. Methylation levels of CG dinucleotides in the promoter region of *ARHGEF12* (OMIM:604763) and *ZNRF3* (OMIM:612062) genes were 12.30% and 10.05% lower in HM cases than in controls, respectively.

Overrepresented Signaling Pathways/Molecular Processes Identified in the Analyses of Genes Overlapping CG Dinucleotides With Decreased Methylation Level

Sixty-five genes with CG dinucleotides having at least a 20% decreased methylation level among cases versus controls were subjected to overrepresentation analyses of signaling pathways and metabolic processes. In the GO and GOrilla enrichment assessments, several overrepresented

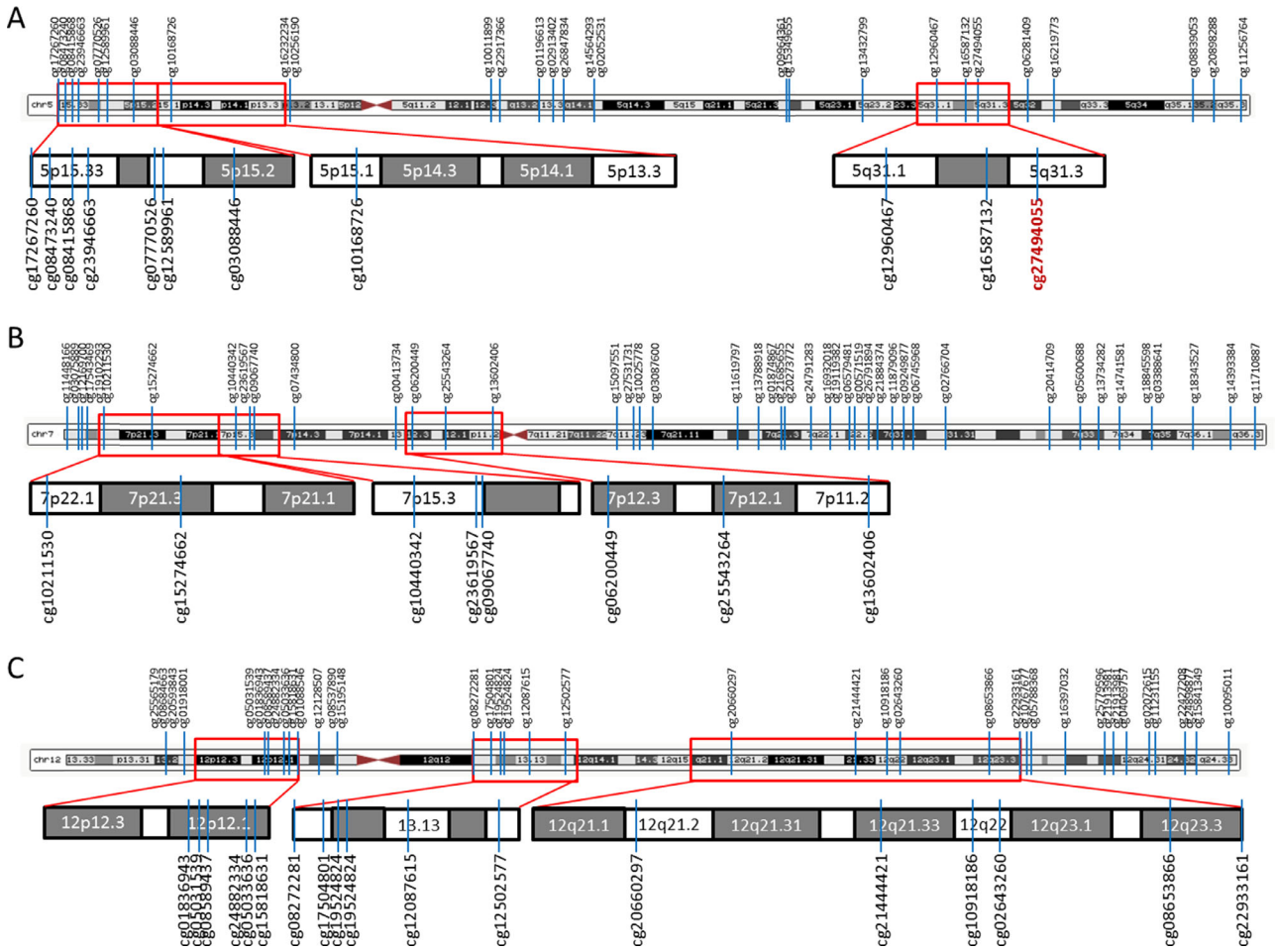

Figure 5. HM/myopia loci at selected chromosomes, with indicated positions of CG dinucleotides with at least a 15% decreased methylation level in children with HM versus controls. (**A**) CG dinucleotides at chromosome 5. Known myopia loci, 5p15.33–p15.2 (MYP16), 5p15.1–p13.3 (MYP19), and 5q31 (MYP25), are marked by a *red frame* and enlarged. cg27494055 in the *PCDHA10* gene is indicated in *red*. (**B**) CG dinucleotides at chromosome 7. Two previously identified HM loci (7p22.1–7p21.1, 7p12.3–7p11.2)[18] and the HM locus 7p15 (MYP17) are marked by a *red frame* and enlarged. (**C**) CG dinucleotides at chromosome 12. Previously identified HM loci 12p12.3–12p12.1[18] and 12q21–q23 (MYP3) and myopia locus 12q13 (MYP24) are marked by a *red frame* and enlarged.

pathways/processes ($q < 0.01$) were predicted, as presented in Supplementary Figure S1 and Supplementary Table S6. Homophilic cell adhesion and cell–cell adhesion via plasma–membrane adhesion molecules were the most significant among the identified. The ConsensusPathDB did not predict significantly overrepresented ($q < 0.01$) pathways or processes.

Discussion

A substantial increase (hypermethylation) or decrease (hypomethylation) in methylation level might influence gene expression, causing silencing or activation, respectively. Alterations of DNA methylation contribute to many disorders, including ocular diseases.[54] Whereas a few studies evaluating DNA methylation in HM have been published, the role of epigenetic modifications in myopia is still not fully recognized.[31–33,36] Previously, we reported that increased methylation level can contribute to the disease in Polish patients.[36] Here, we performed further analyses of our earlier genome-wide DNA methylation data to identify CG dinucleotides with decreased methylation level and the corresponding genes that collectively could contribute to HM pathogenesis in young children with an early-onset HM. By complementing the hypermethylation study with aspects of hypomethylation, we obtained a more complete picture of methylation in children with HM.

Differential methylation of promoter regions may affect gene expression, so such genes were further explored and discussed in the context of involvement in the pathogenesis of HM. Within 55 CG dinucleotides with at least a 20% methylation level difference between cases and controls, only 14 were localized within the 5′ UTR, TSS200, or TSS1500. We found the highest methylation difference between cases and controls (27.07%) for the *PCDHA* gene cluster within a promoter region of the *PCDHA10* gene and in introns of *PCDHA1*, *PCDHA2*, *PCDHA3*, *PCDHA4*, *PCDHA5*, *PCDHA6*, *PCDHA7*, *PCDHA8*, and *PCDHA9* (OMIM:604966). Also, other CG dinucleotides with decreased methylation levels (5.69%–7.86% difference) were found in the intronic sequence of the *PCDHA10* gene. Interestingly, the rs246073, found in introns of most genes of the *PCDHA* cluster, was currently associated with RE in European populations in GWAS ($n = 542{,}934$, $P = 2.0 \times 10^{-14}$).[21] We also detected

this variant in the Polish individuals, but it does not appear to be related to the HM phenotype. Previously, by ES, we identified a nonsense SNV rs200661444 in CpG island in exon 1 of *PCDHA10* in a Polish patient with HM,[26] which was predicted to be disease causing. According to PROMO, rs200661444 overlaps with the human binding site of a transcription factor AP–2alphaA that is required for early morphogenesis of the lens vesicle and regulates transcription of genes involved in eye development. Also, according to PROMO, the presence of SNV disrupts the binding site and prevents binding of transcription factors. In this region, we detected a deletion in members of the Polish family HM-78 and in five children evaluated here, finding no effect of this deletion on the methylation level of cg27494055. Previously, the presence of the deletion was mentioned in Noonan et al.[55]

In spring 2021, the dbSNP database released the SNV variant rs1581364290 that overlaps cg27494055; therefore, all the examined children were genotyped for this variant. The SNV was not detected in any of the samples and did not appear in the HM-78 family; therefore, we ruled out the possibility of a confounding effect of this variant on methylation signals.

On the other hand, by Sanger sequencing, we detected another variant in the *PCDHA* gene cluster, SNV rs251360 G>A in cg27494055. Of note, occurrence of the rs251360 variant creates a potential binding site for the YY1 transcription factor and might cause a decrease in methylation level or demethylation of this site. However, to assess the influence of this variant on gene expression, transcriptome analysis in ocular tissues should be performed.

There are contradictory findings in terms of retinal expression of *PCDHA10*. Whereas transcriptomic results of the ARPE-19 cell line showed only marginal expression of the *PCDHA10* gene, the data in the Human Protein Atlas indicate that the *PCDHA10* gene is expressed in the retina, with the highest expression level in bipolar cells and photoreceptors. These results suggest that due to a complex structure of the retina, the gene might be not ubiquitously expressed in all retinal layers or cells, explaining why *PCDHA10* expression was not detected in the ARPE-19 cell line.

The *PCDHA* gene cluster is one of three related clusters tandemly localized on chromosome 5q31, and it consists of 15 genes and one pseudogene. The 13 upstream genes and the pseudogene have a highly similar sequence, while a subfamily C contains two more distantly related coding sequences (C1 and C2) (Fig. 3). *PCDHA* genes encode neural adhesion proteins that are highly expressed in the brain and likely play a critical role in the establishment and function of specific cell–cell connections in the brain.[56] In the mice study, an impaired *Pcdha* cluster resulted in reduced visual acuity.[57] Moreover, the deletion of *Pcdha* and *Pcdhg* clusters led to more dramatic defects in the survival of inner retinal neurons and dendritic self-avoidance of starburst amacrine cells in mice retinas than *Pcdhg* deletion alone.[58] Other protocadherin genes besides the α cluster were also analyzed in myopia studies. Nallasamy et al.[59] indicated two genes in identified myopia locus 10q21.1 (MYP15) in the Hutterite population from South Dakota, and one of them was *PCDH15* (OMIM: 605514). Moreover, in *Egr1* gene knockout mice with postnatally developed axial myopia, *Pcdhb9* was the most highly differentially expressed retinal gene when compared to wild-type mice.[60] All of these findings point to the *PCDHA* gene cluster, especially the *PCDHA10* gene, as possible candidate genes for myopia/HM.

Transcription factor *ETS1*, another gene that was implicated in this study as probably taking part in HM pathogenesis, regulates angiogenesis in diabetic retinopathy.[61–63] In a mouse model of age-related macular degeneration, intravitreal *Ets1* small interfering RNA alleviates choroidal neovascularization,[64] and GWAS data suggested that *ETS1* was associated with intraocular pressure and glaucoma in Europeans.[20,65–68]

Other genes, *OR6B3* and *FARP2*, are two genes in close proximity to the MYP12 locus. *OR6B3* has been associated with DNA methylation variation in GWAS,[69] supporting the role of methylation in myopia development. We found that *FARP2* had significantly lower CG dinucleotides (20.42%) in its promoter region and within its coding region (14.32%). According to GWAS data, *FARP2* and two other genes, *SORBS2* and *ATXN1*, are associated with educational attainment in Europeans.[70,71] Since university education is a known environmental risk factor for myopia, it could represent a link between epigenetics and environment in HM. *SORBS2* is localized in the MYP22 locus, according to GWAS it is also associated with iris color in South Asians,[72] and mutation in this gene causes abnormal retina morphology in mice (MGI:1924574). The *ATXN1* gene is associated with central corneal thickness among a multiethnic cohort.[73] In summary, the alterations in methylation of the described genes and these genes themselves might provide some insight into the pathogenesis of myopia.

Among the genes overlapping the highest-ranked CG dinucleotides, *ZFAND6* has shown the highest expression in ARPE-19 cells and is located at locus 15q25.1, which has been widely investigated in myopia.[74–76] Moreover, the *ADAM20* gene is at the myopia locus (MYP18), *SBSPON* is associated with corneal curvature/corneal topography in GWAS in Australians,[77] and rs150882242 in *ABHD13* is predicted to be damaging by prediction algorithms. Furthermore, mutations in *Abhd13* and *Lmod3* caused abnormal eye morphology in the mouse model (MGI:1916154, MGI:2444169). Most of the genes indicated in this study are expressed in murine whole eye, retina, and neural retina.[78–81] Again, these data indicate that the mentioned genes may be related to HM in the patients studied.

In enrichment analyses of genes overlapping CG dinucleotides with at least a 20% decreased methylation level in HM patients versus controls, homophilic cell adhesion and cell–cell adhesion via plasma–membrane adhesion molecules were predicted as the most significant. Nervous system development, cell–cell and biological adhesions, and developmental process were also highlighted. Again, in most predicted biological processes, the genes from the *PCDHA* family were found to be involved, with the already mentioned differentially methylated CG dinucleotide as a dominant and potentially causative factor.

We also compared our results to other methylation studies in myopia. Previously, the decrease in DNA methylation was linked to a higher risk of early-onset myopia.[31] Several CG dinucleotides identified as hypomethylated in umbilical cords of 29 myopic children of Chinese, Malay, or Indian origin from Singapore (with myopia diagnosed later at the age of 3 years), when compared to 490 matched controls in Seow et al.,[31] were not located in promoter regions and did not reach significant differences in methylation level in our study. In a study on chicks, the ocular growth development was not associated with substantial changes in DNA

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methylation, but significant changes in methylation levels at single CpG sites were found in the studied *EGR1*, *FOS*, and *NAB2* genes.[35] This supports our findings as we observed significant changes at individual CG sites, rather than the large-scale shifts, although at different genes. In another study, six CG dinucleotides in the promoter and exon 1 region of *Col1a1* were hypermethylated, and scleral *Col1a1* messenger RNA levels were reduced in the treated mouse eyes with monocular form-deprivation myopia (FDM) compared with normal murine control eyes,[32] suggesting that DNA methylation of the *Col1a1* promoter/exon 1 may inhibit the synthesis of scleral collagen leading to myopia development.[32] Although the *COL1A1* gene has been studied frequently as a candidate gene for HM, the outcomes are still inconsistent.[82–88] Significantly lower methylation of four CG sites in the *IGF1* gene promoter and moderately higher transcription level in the sclera were indicated in a guinea pig model of FDM, which suggest the role of this gene methylation in FDM pathogenesis.[34] We observed only nonsignificant changes in methylation levels of CG dinucleotides within this gene.

To our knowledge, this is the first report showing decreased DNA methylation level of CG dinucleotides in Polish children with HM. However, further analyses of a larger cohort, including assessments of children's lifestyle and other environmental factors, are needed. Moreover, we did not examine whether the children's mothers were exposed to pollution, heavy metals, smoking, or nutritional habits during pregnancy, as these are well-known factors affecting methylation in children.[89] The study limitation is also the assessment of DNA methylation patterns in blood samples instead of eye tissue as it was impossible to obtain retinal samples from the ascertained children. However, other methylation studies were also performed on the blood of patients with HM rather than on the target tissue, making all results comparable.[33] Functional analyses were beyond the scope of this project.

Conclusions

In summary, differential methylation of identified candidate CG dinucleotides might play a role in HM pathogenesis in the examined patients. Alterations in the methylation levels of promoter regions of *PCDHA10*, *ADAM20*, *PAG1*, *ZFAND6*, *ETS1*, *ABHD13*, *LIG4*, *SBSPON*, *SORBS2*, *SLC25A3P1*, *TANC1*, *LMOD3*, *ATXN1*, *FARP2*, and *OR6B3* might affect the expression of these genes, lead to disruption of their function, and thus contribute to the HM phenotype. Still, the role of *PCDHA10* gene methylation and sequence variants in this gene remains inconclusive. Broadening the knowledge on the epigenetic basis of HM will increase the understanding of the molecular mechanisms of HM and other eye diseases. Changes in methylation patterns of specific CG dinucleotides in children could serve as potential noninvasive biomarkers for HM/myopia. Moreover, identifying additional factors that influence the eye phenotype is a step toward improving molecular diagnostics and better treatment in HM. Overall, the obtained results support the role of DNA methylation in HM pathogenesis.

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