The End of Myopia

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Abstract

Several haplotypes of the cone opsin genes, *OPN1LW* and *OPN1MW*, cause exon 3-skipping during pre-messenger RNA splicing and are associated with high-myopia (nearsightedness). Cone photoreceptors expressing these haplotypes have substantially less photopigment than cones in the same retina expressing non-exon-skipping haplotypes. Thus, we hypothesized that abnormal contrast signals arising from adjacent cones with different amounts of photopigment stimulate axial elongation of the eye, causing myopia. If so, opsin gene haplotypes exhibiting milder exon-skipping might be associated with common juvenile-onset myopia, and it may be possible to use contrast-reducing spectacles to slow myopia progression. We present experimental evidence that opsin gene haplotypes are major risk factors for juvenile-onset myopia rivaling all other known polymorphisms combined. Moreover, contrast-reducing lenses significantly slowed axial elongation of eyes of myopic children, thereby slowing the progression of myopia. Combined, contrast-reducing spectacles and early intervention through genetic identification of myopia-susceptible children illuminate a pathway to end myopia.

Introduction

Myopia is a leading cause of blindness^{1,2}, and its prevalence is rising²⁻⁴. Genetic and environmental factors contribute to excessive elongation of the eye, which causes myopia⁵. Despite intensive searches for myopia genes in genome-wide association studies (GWAS), identified markers explain only ~18.4% of the heritability and 12.1% of the variance in spherical equivalent refraction (SER)⁶, with the largest effect size for a single nucleotide polymorphism (SNP) being only ~0.15 diopters (D)⁷⁻⁹. Moreover, results have not translated into new prevention strategies.

The human long-wavelength (L) and middle-wavelength (M) cone opsin genes (*OPN1LW* and *OPN1MW*, respectively) exhibit high haplotype diversity¹⁰⁻¹². High-myopia-associated haplotypes¹³⁻¹⁷ induce 80-100% exon 3-skipping *in vitro*¹³⁻²⁰ thus, we looked for an association between weaker exon 3-skipping haplotypes and common juvenile-onset myopia by sequencing the *OPN1LW* gene and estimating SER from axial length and corneal curvature measurements for males with normal colour vision. Such males typically have one *OPN1LW* followed by one or more *OPN1MW* genes, but only the *OPN1LW* gene and the adjacent *OPN1MW* gene are expressed^{12,21,22}. We report that *OPN1LW* SNPs have the largest myopic refractive error effect sizes associated with common juvenile onset myopia ever described.

The first high-myopia-associated opsin gene haplotype, abbreviated LVAVA for the deduced amino acids at exon 3-encoded amino acid positions 153, 171, 174, 178, and 180 (Leucine 153, Valine 171, Alanine 174, Valine 178, Alanine 180)^{13,23,24}, impairs splicing, resulting in cones that are nearly "empty" of photopigment^{15 16,18}. High-myopia is characteristic of males expressing the LVAVA haplotype in one cone submosaic and expressing another opsin gene haplotype with relatively unimpaired splicing¹⁵ that produces photopigment-filled cones in a second submosaic. Abnormal contrast signaling between neighboring full and empty cones may stimulate axial elongation. If so, contrast-reducing spectacles

could potentially slow axial growth. We describe a pilot study showing that contrast-reducing eyeglasses effectively slowed the axial elongation rate in myopic children. An optimized version of the spectacles reduced myopia progression by 74% after one year in a multisite clinical trial (ClinicalTrials.gov Identifier: NCT03623074)²⁵. Combining opsin genetics and contrast-reducing eyeglasses provides a rational approach to control eye growth safely and effectively with the potential to end myopia.

Results

The relationship between Xq28 opsin gene haplotypes and refractive error

The relationship between SER and *OPN1LW* haplotypes was evaluated using 413 adult males with normal color vision^{26–28} (see methods). Sequences and relative numbers of *OPN1LW* and *OPN1MW* genes were determined^{26,28}. We calculated each subject's SER from axial lengths and corneal curvatures (see Supplementary Data) using the equation derived in Supplementary Figure S1. There were eleven haplotypes among the 413 participants (see Supplementary Data), and the median SER varied significantly more than expected by chance ($p = 0.0082$, Kruskal-Wallis test), suggesting a role for *OPN1LW* polymorphism in juvenile-onset myopia; however, this analysis did not identify specific myopia-susceptible haplotypes. To get at this, we tested for an association between calculated SERs and the exon 3 SNP that most significantly impaired splicing in a minigene splicing assay. The percentage of exon 3-skipped mRNA was measured for 128 minigenes with different exon 3 haplotypes (Supplementary Table S1). Splicing was most impaired for exon 3 haplotypes with G at SNP rs145009674 in codon 178 (Fig. 1), which skipped exon 3 on average 12.7 times more than haplotypes with A ($p < 0.0007$, Mann Whitney U after correcting for multiple comparisons). SNPs rs155715655 in codon 180 and rs5986963 in codon 171 also significantly impaired exon 3-inclusion, but to lesser degrees (Fig. 1).

Comparing the SERs calculated for subjects with A ($n = 401$) versus G ($n = 12$) at rs145009674 revealed a median SER of $-0.9975D$ for individuals with A and $-2.920D$ for individuals with G; thus, the median effect size was $\sim 2D$ ($p = 0.005$, Mann Whitney U). The mean calculated SER for the two groups differed by $1.36D$ (-1.1486 vs. -2.504), an effect size 13 times larger than any previously reported SNP associated with a negative dioptric change^{6,9}, clearly establishing SNPs in *OPN1LW* as significantly associated with juvenile-onset myopia. The population frequency of G at SNP rs145009674 is $\sim 2.5\%$ and among Europeans it is 2.07% ²⁹. In our sample, the calculated SER was worse than $-0.75D$ for 82% of individuals with G at rs145009674. Reportedly, 30 out of 100 Europeans are myopic³⁰. If two or three of 100 Europeans have G at rs145009674, 82% of them (1.7 to 2.5 people) with the G allele will be among the 30 myopes. Thus, having G at rs145009674 accounts for about 6–8% of myopes. The combination of a relatively very large effect size (about 2D) and high frequency in the population makes this single SNP, by far, the most significant single determinant of juvenile-onset myopia ever discovered. Compare this with the fact that all previous SNPs ever discovered together account for only about 12% of refractive error^{6,9}.

We noted above that two other SNPs also are associated with significant exon 3 skipping. The median SERs for the eleven common haplotype groups varied more than expected by chance, and the mean SERs ranged over more than 4D (Table 1). This suggests that the *OPN1LW* haplotypes are responsible for more refractive error than is evident just from our analysis at SNP rs145009674, but how much?⁹ About half of the refractive error SNPs discovered by GWAS are associated with myopia, accounting for ~6% of the myopic variance. We estimated the variance in SER attributable to *OPN1LW* polymorphisms by ranking the myopia risk of the haplotypes using a split halves analysis (Table 1, see methods). The mean refractive errors of the haplotypes were strongly correlated to the final haplotype ranks (Supplementary Fig. S2) with a coefficient of determination (r^2) of 0.98; the probability of this occurring by chance is 4.51×10^{-9} . Haplotype rank can be used as a measure of the relative risk an individual has for becoming myopic. Thus, the association between SER and haplotype rank for individual subjects is a gauge for the amount of variance in myopia attributable to the *OPN1LW* haplotypes; the coefficient of determination for this association is 0.046, indicating polymorphisms in *OPN1LW* account for about 4.6% of the variance in juvenile-onset myopia.

With our sample size, we could only analyze the eleven highest frequency *OPN1LW* haplotypes; however, low-frequency *OPN1LW* haplotypes and *OPN1MW* haplotypes are likely to contribute to myopia through a similar mechanism. For example, in our sample males with *OPN1MW* genes encoding only MVVVA ($n = 64$) versus everybody else ($n = 349$) differed in average SER by 0.39 D (Supplementary Data). Adding this single *OPN1MW* polymorphism to the opsin risk calculation raises the r^2 , and thus the amount of variance explained by polymorphisms at the X-chromosome opsin gene locus to 4.9%. Determining how much of the variance in SER is attributable to the full spectrum of haplotypes at the *OPN1LW/OPN1MW* locus would require a larger sample; however, this study revealed that haplotypes of these genes are the major heritable risk factor for myopia and the primary source of heritability unaccounted for in GWAS.

We hypothesized that exon3-skipping haplotypes reduce the amount of photopigment in cones, thereby activating contrast pathways between adjacent cones and stimulating the eye to grow excessively³¹. If so, people with skewed ratios of L to M cones (L:M) should be relatively protected from myopia because cones with different abilities to catch photons are less likely to be adjacent to each other. Red-green colour blind individuals have the most skewed cone ratios, having 100% L or 100% M cones, and are relatively protected from myopia³². We compared colour normal subjects with skewed cone ratios to those with more balanced proportions. The average cone ratio in people of European ancestry is about 2L:1M^{33,34}. Thus, we determined the cone ratios in 357 of the 413 subjects (Supplementary Data) using previously described methods³³ and split the sample into a "skewed group" ($\geq 67\%$ and $\leq 33\%$ L cones, $n = 164$) and a "more balanced group" ($\leq 66\%$ and $\geq 34\%$ L cones, $n = 193$). The skewed group had on average 0.61D less myopia ($p = 0.006$) than the balanced group. Having a more balanced ratio of L to M cones is, thus, a significant risk factor for myopia, providing strong support for the contrast hypothesis.

We studied males but expect a significant amount of myopia is associated with *OPN1LW* haplotypes in females; however, demonstrating this requires a larger sample size because females have two X-

chromosomes while males have one. The contrast theory predicts that *OPN1LW* haplotypes will manifest as X-linked dominant for myopia, consistent with reports that female carriers of the LVAVA cone opsin variant are myopic^{14,24}. More generally, the incidence of myopia, particularly progressive high-myopia, is higher among females³⁵. As an example of how a dominant inheritance pattern of myopia-susceptible opsin alleles might manifest in females, the frequency of G at rs145009674 in *OPN1LW* is 2.1%²⁹. Hence, males have a 2.1% chance of having one myopia allele, but females have a 4.1% chance of having one copy and 0.044% chance of having two copies. Thus, the cone mosaics will be different for males versus females with one mutant allele. People of European ancestry, on average, have a ratio of 2L:1M cones; therefore, males will have two mutant cones for every normal one. Females with one myopiagenic *OPN1LW* haplotype, and one non-myopiagenic *OPN1MW* haplotypes will have, on average, two normal cones for each one expressing the myopia-susceptible allele. However, according to the contrast theory, the frequency of mutant and normal cones adjacent to one another is important, not the number of cones expressing the myopia-susceptible allele. Thus, males and females who inherit one myopia-susceptible allele can be similarly affected.

As shown in Table 1, some haplotypes with the highest myopia risk do not have high exon 3-skipping values; this is not unexpected. For example, MVVIS exhibits 0% exon-skipping, but 13 of 23 subjects with this haplotype have an *OPN1MW* exon haplotype (MVVVA or MVAVA) that displays $\geq 16\%$ exon 3 skipping. Thus, the high-myopia risk associated with *OPN1LW*_{MVVIS} may be due to its tendency to occur with exon-skipping *OPN1MW* haplotypes. In addition, genetic polymorphisms affect both the splicing and amino acid codes. Altering the amino acid sequence affects opsin function, which may produce contrast between adjacent cones expressing different haplotypes¹⁶.

Why was *OPN1LW* not identified in GWAS searches for myopia genes^{7,36-40}? The failure is not surprising given that the *OPN1LW*_{LVAVA} haplotype's role in myopia was overlooked for over ten years despite direct mapping of MYP1 to Xq28^{24,41-46}. Moreover, few GWAS included the X-chromosome, none included opsin gene SNPs, and GWAS relies upon high linkage disequilibrium between marker SNPs and disease-causing mutations⁴⁷, but *OPN1LW* exon 3 exhibits a high recombination rate and lower-than-expected linkage disequilibrium¹².

Myopia prevention using spectacles that control the spatial distribution of light reaching the retina.

The association between high-myopia and exon-skipping in the Xq28 opsin genes led to the hypothesis that increased contrast signaling is the common pathway in myopia development and progression. We tested the theory by measuring the effect of reducing retinal contrast on axial elongation in children with progressive myopia. To maximize statistical power and minimize sample size, each participant wore an experimental lens on one eye and a control lens on the other eye. Both lenses were standard of care single vision lenses. The experimental lens had a red light blocking tint and light scattering elements that spread incident light over a 0.5-degree angle, reducing contrast and the relative activity difference between L and M cones that results from visual experience. The best-corrected acuities of participants

ranged from 20/15 to 20/20 without light scattering and 20/20 to 20/25 with light scattering. The control lens had a neutral tint, so both eyes experienced the same luminance, removing it as a variable. Participants had progressive myopia, documented by a change in refractive error of at least -0.5D during the previous year. Thirteen subjects wore the experimental lens on their dominant eye and the control lens on the non-dominant eye. After three months, seven participants re-enrolled for another three months, during which they wore the experimental lens on the non-dominant eye and the control lens on the dominant eye. Supplementary Table S2 lists the baseline characteristics of participants. The primary outcome measure was the change in axial length from baseline for the eye wearing the experimental lens versus the control lens over three months. As predicted by the contrast theory of myopia, Fig. 2 shows that there was a dramatic reduction in average axial length growth rate in the eyes wearing the experimental lens ($0.063 \pm 0.33 \mu\text{m/day}$; mean $\pm$ SE), versus control ($1.43 \pm 0.24 \mu\text{m/day}$) and the difference was highly statistically significant ($p = 0.0019$).

Discussion

The nature of the opsin mutations associated with high-myopia and juvenile-onset myopia support the contrast theory of myopia, as does the observation that skewed L:M cone ratios protect against myopia. Finally, all known optical devices that effectively slow the progression of myopia project multiple optical powers onto the retina, creating blur, which reduces contrast^{48,49}.

The eyeglasses study described here directly tested and supports the hypothesis that contrast stimulates the eye to elongate, causing myopia. Based on these encouraging results, we subsequently optimized the lens design for efficacy, tolerability, and long-term wear by children. Optimizations included improving the method for incorporating durable light scattering elements, adding a ~7mm clear central aperture, and excluding the tint⁵⁰. These lenses are now the subject of a controlled, multi-center clinical trial in which 256 myopic subjects were randomized at 14 clinical sites and dispensed lenses comparing myopia progression in children wearing standard-of-care lenses versus two different contrast-reducing lenses that differ in the density of light scattering elements (ClinicalTrials.gov Identifier: NCT03623074). The 12-month interim analysis results demonstrated superiority for cycloplegic SER change from baseline ($P < 0.0001$) for both test arms. One test arm reduced myopia progression by 74%, the other reduced it by 59%^{25,51}. This validates the effectiveness of contrast reduction in reducing myopia.

Young children are far-sighted⁵²; thus, distant scenery filling peripheral vision is highly focused, producing high contrast and stimulating eye growth. The distant scenery goes out of focus as the eye elongates, lowering contrast and reducing the stimulus to elongate. The balance between contrast and growth stabilizes at emmetropia when the eye achieves the optimal length for its focusing power. This mechanism worked well in outdoor environments where humans evolved, but not in modern environments where near-work, such as reading and screens, fills peripheral vision with high-contrast images, stimulating eye growth, particularly in genetically predisposed children.

Ironically, the contrast theory of myopia contradicts the orthodox view that blur stimulates eye growth. How can these theories be reconciled? The blur hypothesis is based mainly on form-deprivation experiments^{53 54,55}, in which strong diffusers on animal eyes in laboratory settings cause myopia. However, Smith et al.⁵⁶ recently investigated the effect of brighter outdoor lighting on eye growth. Nonhuman primates wore diffusers under conditions where auxiliary lighting raised the light levels from 15–630 lux to ~25,000 lux for 6 hours during the middle of the daily 12-hour light cycle. Most animals tested under these conditions developed form-deprivation *hyperopia*. Bright sunlight is usually above 100,000 lux; thus, diffusers usually produce a hyperopic shift in primates under light levels that approach natural conditions, as predicted by the contrast hypothesis.

Contrast-gain-adaptation may explain why strong diffusers cause myopia at low light levels. The visual system increases its contrast gain depending on the amount of contrast on the retina⁵⁷. Wearing strong diffusers may increase the gain until noise is transmitted as real contrast. Higher light levels may reduce noise so that strong diffusers produce hyperopia.

Mild hyperopia, the natural state of refractive development in young children, is outgrown as the eye elongates during emmetropization⁵². Myopia researchers argued that this fit with the form deprivation results under the logic that, when looking in the distance, hyperopia would blur images on the retina^{58,59}. However, this overlooks three facts. First, local retinal mechanisms mediate ocular growth in primates, and the peripheral retina is the primary mediator of emmetropization⁶⁰. Second, under natural outdoor conditions in which humans evolved, faraway scenery fills our peripheral vision. Third, distant scenes are focused and produce high contrast in far-sighted children because their tonic accommodation off-sets their short axial lengths. Thus, consistent with the contrast hypothesis, peripheral contrast is reduced in the tonically accommodated eye as it grows, reducing the signal for further growth.

To the extent that the contrast hypothesis proves correct, it can be the basis for a new rational, mechanistic approach to ending myopia. Identifying myopia-susceptible children through genetic testing and intervening early with spectacles that reduce myopia progression by 74% may allow the elimination of high-myopia, in turn reducing the risk for vision-threatening eye conditions that are secondary to myopia. These include retinal detachment, glaucoma, cataracts, and myopic macular degeneration⁵. Modeling predicts that, without effective intervention, a billion people on earth will have high-myopia by 2050^{2,61}. With continued research to better identify myopia-susceptible children and improve spectacle lens designs, it should be possible to end myopia.

Methods

Cell lines: Authenticated HEK 293T (female) cells (RRID: CVCL_0063) were obtained from ATCC. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 2mM L-glutamine and maintained at 37°C with 95% carbon dioxide.

Human Subjects Institutional Review Boards approved human subjects research at the University of Washington, Medical College of Wisconsin, and Children's Wisconsin Hospital-Milwaukee. Research

involving human subjects was performed in accordance with local and federal regulations. Informed consent was obtained from all participants and their legal guardians. Three separate groups of subjects were involved in experiments. First, we recruited 373 adult subjects at MCW (Supplementary Fig. S1). These subjects provided their most recent refractive error measurement from their optometrist or ophthalmologist. Study procedures for these subjects included colour vision testing using the Richmond HRR pseudoisochromatic plates 4th edition and measurements of axial lengths and corneal curvatures using the Zeiss IOL Master. We derived an equation that calculates each participant's spherical equivalent refraction (SER) from the IOL Master data (Supplementary Fig. S1).

We recruited a second group of ~1,000 adult males at UW and evaluated the relationship between their opsin gene haplotypes and SER calculated from IOL Master measurements of axial lengths and corneal curvature using the equation described in Supplementary Fig. S1. In addition to the study procedures described above for the 373 subjects at MCW, the UW cohort also provided blood or saliva samples for DNA extraction. We sequenced the X-chromosome cone opsin genes²⁶ and estimated the relative number of *OPN1LW* and *OPN1MW* genes on the X-chromosome for each subject using the Agena MassArray genotyping system²⁸. Electroretinography (ERG) was performed, and the ratio of L to M cones for each subject was estimated from the ERG data and genetic data, using previously described methods¹¹. Subjects reported their ancestry. Individuals with new or rare mutations in the *OPN1LW* or *OPN1MW* genes were excluded from the haplotype analysis. We included subjects with one *OPN1LW* gene sequence and with an *OPN1LW* haplotype shared by at least four participants. Ultimately 413 of these subjects fit the inclusion criteria for the analyses looking for an association between SER and *OPN1LW* gene haplotype (Table 1, Supplementary Fig. S2, Supplementary Data).

We tested contrast-reducing eyeglasses for their effect on myopia progression in thirteen children recruited at MCW (Figure 2). We obtained approval for the eyeglasses study from the IRB at MCW and Children's Wisconsin Hospital – Milwaukee. Procedures performed on participants included colour vision testing with the HRR Richmond 4th edition pseudoisochromatic test, axial lengths, and corneal curvatures measurements made using the Zeiss IOL Master. Participants (n = 13) were referred by eye care practitioners who knew the eligibility requirements. Thus all subjects we assessed were eligible. The thirteen participants enrolled for three months and were allowed to re-enroll for a second three months. Seven participants elected to re-enroll for the second three months of the study. The characteristics of each participant are listed in Supplementary Table S1. All subjects completed the first three months, and 7 completed a second 3 months. No participant withdrew from the study. All data from all subjects was included in the analysis (Figure 2).

Axial lengths, corneal curvature, and estimated spherical equivalent refraction (SER): Axial lengths and corneal curvatures were measured for each subject using the Zeiss IOL Master, and the predicted spherical equivalent refraction (SERs) were calculated using a formula derived from a linear regression of a dataset of observed SERs, axial lengths (AL) and corneal curvatures (CC) from a group of 373 subjects (Figure 1). The formula is: $SER = -(AL * 2.03 + 0.94 * CC) + 88.58$, where the value for AL was the average

of 20 measurements per eye, and CC was the average of the two different methods of measuring read out from the IOL master.

Minigenes: We created minigenes using the procedure described by Ueyama et al.¹⁸. Minigenes contained a full-length human *OPN1LW* cDNA with the full-length introns 2 and 3 inserted on either side of exon 3. Site-directed mutagenesis was performed using the QuickChange kit (Agilent) or the In-Fusion system (Clontech) to create 128 different exon 3 haplotypes. Individual exon 3 haplotypes were cloned into the minigene using the *Hind*III and *A*flI sites flanking exon 3. The 128 minigenes generated were sequence-verified, and the only differences among them were the sequences of exon 3. Exon 3 haplotypes varied at eight nucleotide positions, described here using SNP ID numbers. rs949930 was A or G, rs713 was A or C, rs731614 was C or G, rs5986963 was A or G, rs5986964 was T or G, rs149897670 was C or T, rs145009674 was A or G, and rs155715655 was G or T. Minigenes represented all combinations of these SNPs except rs5986964. Typically in *OPN1LW* genes, rs5986963 and rs5986964 are A and T, or G and G, so these are the only combinations represented in the 128 minigenes. Exons 2 and 4 of the human *OPN1LW* gene are also polymorphic. The minigenes all had the following residues at the variable positions: c.194C (rs782815809) c.300A (rs1065420), c.331A (rs1065421), c.362C (rs1065422), c.689T (rs148583295), c.697G (rs781936473), c.698C (rs1292153674), c.699T (rs1226772901), c.706A (rs1065426).

Splicing Assays

Using the procedure described by Ueyama et al.¹⁸, 1.1 µg of each minigene plasmid was transfected into duplicate cultures of HEK293T cells using Fugene HD. Forty-eight hours after transfection, RNA was isolated, reverse transcribed using the Superscript First-Strand Synthesis kit (ThermoFisher), and primed with oligo dT. The resulting cDNA was used in a genotyping assay employing the Agena MassArray genotyping platform to estimate the percentage of cDNA lacking exon 3 for each of the 128 exon 3 haplotypes.

The MassArray Assay The MassArray genotyping platform was used for the quantitative assessment of exon 3-skipping. The assay was first validated by creating a standard curve using known ratios of DNA templates with exon 3 included and exon 3 excluded. Genotyping was performed using the Agena iPLEX Pro kit according to the manufacturer's instructions. Briefly, cDNA was subjected to PCR amplification and primer extension using the iPLEX Pro PCR reagents. Assays, including primers, were designed using the manufacturer's software. A cDNA segment extending from exon 2 to exon 4 was PCR amplified with a forward primer to sequences in exon 2, starting 73 bp upstream of the 3' end of exon 2 (primer sequence 5' ACGTTGGATGAACCAGGTCTCTGGCTACTT), and a reverse primer to sequences in exon 4 starting 82 bp downstream of the 5' end of exon 4 (reverse primer sequence 5' ACGTTGGATGAATCTCACATTGCCAAAGGG). The underlined bases in each primer were added to remove the primers from the mass window observed in the analysis of the extension products by the MassArray MALDI-TOF mass spectrometer. The PCR reactions were treated with iPLEX Pro Shrimp Alkaline Phosphatase to dephosphorylate the unincorporated dNTPs. The PCR products were then subjected to a

single nucleotide primer extension reaction using the iPLEX Pro Extend reagents. The extension primer sequence was 5' AGGCCGTGGGGCCAGTACC which corresponds to the 5' end of exon 4 extending toward exon 3 with the last nucleotide of the primer corresponding to the 3'most nucleotide of both exons 2 and 3. The extension reaction incorporates a T residue if exon 4 is spliced to exon 3, or an A residue if exon 4 is spliced to exon 2 and exon 3 is skipped. Extension reactions were carried out with mass modified terminator dNTPs so that the T vs A difference in the extension products is sufficient to resolve them by MALDI-TOF mass spectrometry. Desalting of the extension reactions was accomplished by adding iPLEX Clean Resin, followed by spotting reaction products onto a SpectraChip (Agena), and analyzed with the MassArray MALDI-TOF mass spectrometer. Note, if intron 2 had been retained, the extension product would be the same as that expected if exon 2 was spliced to exon 3 and would lead to an underestimate of the amount of exon 3 skipped. However, PCR products were observed by agarose gel electrophoresis and subjected to Sanger Sequencing. The major products were correctly spliced and exon 3-skipped; thus, intron retention is unlikely to affect the results significantly. The areas under the curves (AUC) for each of the extension products were used to calculate the percentage of mRNA with exon 3 skipped ($\% \text{ exon 3 skipped} = 100 * (\text{Area under A extension product}) / (\text{area under A extension product} + \text{area under T extension product})$). The percentage of exon 3-skipping for each haplotype was taken as the average of the number of biological replicates performed, typically two replicates (see Supplementary Table S1). The within-group standard deviation is typically 1.5% or less with this assay, thus using duplicate biological replicates provided 80% power to detect a 5% difference in exon 3-skipping with a p-value of 0.05.

Statistics

GraphPad InStat 3.06 was used to perform statistical analyses, except for the split-halves analysis which was done using a custom MATLAB script. The split halves analysis was performed using data from the 413 males recruited from UW who met the study inclusion criteria in the Methods. They were randomized into two groups. Haplotypes in each group were ranked according to their average refractive errors with haplotype rank 1 having the most myopic refractive error and rank 11 having the least myopic refractive error. If the absence of an association between haplotype and refractive error, the correlation between rank and refractive error would not be statistically significant. Because the results for any two halves are independent, the correlation between ranks for the two halves would also not be statistically significant. To obtain the most accurate ranking possible for the haplotypes, we repeated the random draw ranking 1,000 times then averaged the rankings of the 1,000 randomized halves. We validated the procedure by repeating the process with the same subjects and the same haplotype group sizes but we randomly scrambled the assignments of the subjects to haplotype groups. This yielded a non-significant p-value of 0.44 and a low r^2 of 0.07, demonstrating that the procedure does not produce a significant correlation artifactually.

Declarations

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All authors contributed to data interpretation. Manuscript was written by MN, JN and incorporated comments by all others.

Data Availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Additional Information:

JN, MN and JAK receive royalties and licensing fees related to the eyeglasses through MCW and UW. MWS and JSR have no competing financial interests.

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Tables

Table 1
OPN1LW exon 3 haplotypes tested for associate with SER

Haplotype ¹	Amino Acids ²	N ³	Median/Mean SER OS ⁴	% Exon 3-skipping	Split Halves Rank ⁵
AACGGTGG	MVVVA	12	-3.16/-3.14	16	1
AACGGCAT	MVAIS	30	-1.62/-2.00	3	2.49
AACGGTAT	MVVIS	23	-2.01/-2.57	0	2.51
AACGGCAG	MVAIA	67	-1.05/-1.71	2	4.25
GCGGGCAG	LVAIA	77	-0.97/-1.38	14	4.75
GCGGGCAT	LVAIS	157	-0.99/-1.38	1	6
GCGATCAT	LIAIS	25	-1.10/-1.30	1	7
AAGGGCAT	MVAIS	9	+0.15/+0.51	0	8.99
GCGGGGAT	LVVIS	5	-1.06/-1.04	1	9
GCCGGCAT	LVAIS	4	-.05/-0.25	8	9.02
GCCGGCAG	LVAIA	4	+0.54/+0.92	2	11
¹ Haplotypes are nucleotide identities for dbSNP ID numbers rs94930, rs713, rs731614, rs5986963, rs5986964, rs149897670, rs145009674, and rs155715655, in that order.					
² Amino acids indicated at protein positions 153, 171, 174, 178, 180 using the single letter amino acid code (M = methionine, V = valine, L = leucine, A = alanine, I = isoleucine, S = Serine.)					
³ Number of subjects with each haplotype.					
⁴ Median and mean Spherical Equivalent Refraction (SER) for subjects with the indicated haplotype are separated by a slash. The SER was calculated for the left eye (OS) for each subject from axial length and corneal curvature measurements made with the Zeiss IOL Master (see Supplementary Data) using the formula given in Methods and described in Supplementary Figure S1.					
⁵ Ranks are those determined in the split halves analysis, see Supplementary Fig. S2.					

Figures

Figure 1

Three of seven exon 3 SNPs differ significantly in exon skipping.

The nucleotide sequence of *OPN1LW/OPN1MW* exon 3 is given. The eight polymorphic nucleotides analyzed in this study are shown in red letters using the IUB code where R is A or G, M is A or C, S is G or C, K is G or T, and Y is T or C. Black triangles underscore the codons containing the SNPs and the codon numbers are indicated. More information about each SNP can be found at in db SNP (<https://www.ncbi.nlm.nih.gov/snp/>). Below the codon numbers are the two alternate amino acids coded, below which are the two common nucleotides at each SNP. At each position, the amino acid and nucleotides are given as allele 1/allele 2. For example, codon 151 is either AGG or AGA, designated G/A. Both of these codons encode arginine, designated R/R using the single letter amino acid code. Codon 153 is either CTG or ATG, designated C/A and encodes leucine or methionine, respectively, designated L/M (see Table 1 for the single letter amino acid code). SNP ID numbers in the left-most column are from dbSNP151 for the *OPN1LW* gene. The Mann Whitney U results for the median percentage of exon 3-skipping for each SNP except the third nucleotide of codon 171 (labeled na for not applicable) are in the right-most column. A slash (/) separates values for alleles 1 and 2. Bonferroni corrected p values for each SNP's effect on exon 3-skipping are indicated except where the value was not significant (designated n.s.).

Figure 2

Normalized axial length measurements as a function of time. Thirteen dominant eyes wore the experimental lens (right), and 13 fellow eyes wore the control lens (left) for three months. Seven subjects re-enrolled for a second three-month period, during which the non-dominant eye wore the experimental lens and the dominant eye wore the control lens. Myopia progression significantly slowed in both groups (13 eyes and 7 eyes) when the two groups were analyzed separately. The combined data are shown. Each coloured line represents measurements for one eye. Lines of the same colour in the right and left plots represent data from the same subject. Black lines are averages for all eyes (error bars ± 2 SEM). The experimental lenses significantly reduced the rate of eye growth of myopic children ($p = 0.001$). Each data point is an average of twenty measurements.

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