Journal of
Emerging Investigators

TGFβ1 Codon 10 Polymorphism and its Association with the Prevalence of Low Myopia

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Summary

The goal of this project was to assess the relationships among low myopia, behavioral and demographic factors, and a single-nucleotide polymorphism (SNP) in the TGFβ1 gene that codes for an extra-cellular matrix (ECM) protein previously linked to scleral growth. This research study adds a unique perspective as it provides clarity to the inconclusive relationship between low myopia and TGFβ1, and it is the first such association study to include a racial demographic outside of Asia. Genetic data was obtained using real-time PCR, and behavioral and demographic data were acquired through surveys. Genetic results indicated that the variant C allele, and in particular the TC genotype, is associated with the prevalence of low myopia. It was further concluded that the C allele is associated with a greater severity of low myopia and that Asian Indians and East Asians are more likely to have the variant C allele than those of European ancestry. The study also found that environmental factors such as increased reading time, decreased non-stationary exercise, overall reading distance, and other factors are associated with an increased prevalence of myopia. These findings could be helpful in behavioral modification in the prevention of myopia. Additionally, they confirm that both environmental and genetic factors are involved in the development of myopia. Knowing if a person has a genetic polymorphism that would predispose them to myopia could encourage more aggressive behavioral management to prevent its development.

Received: January 24, 2017; **Accepted:** June 23, 2017; **Published:** December 11, 2017

Introduction

Myopia (nearsightedness) is a common cause of blurred vision. An estimated 23% of the world's population has myopia (< -0.25 diopters) and 2.7% has high myopia (< -6.25 diopters) (1). Myopia is measured on a discrete scale in increments of 0.25 diopters, with the diopter being a unit of measure of the power of the lens needed to correct vision to normal. Myopia is often caused by axial elongation, or lengthening of the eye. Formation and remodeling of the sclera, the white outer layer of the eyeball, has been linked to axial elongation (2). Scleral remodeling is associated with the quality and quantity of extracellular matrix (ECM) proteins. Extracellular matrix proteins provide support and structure to the cell. Thus, altered ECM proteins could be linked to deteriorated architecture of the sclera (3).

One recent area of genetic study regarding myopia and ECM proteins involves polymorphisms of the transforming growth factor beta one (TGFβ1) gene. TGFβ1 has been implicated in a wide variety of traits, from chronic pancreatitis to breast cancer to myopia. TGFβ1 plays a role in the production of the ECM proteins involved in scleral construction. A single-nucleotide polymorphism known to occur in one nucleotide of codon 10 in the TGFβ1 gene (SNP ID# rs1800470) changes the codon's translation of a leucine to a proline amino acid and thus alters the overall shape of ECM proteins (4).

Lin *et al.* (5) evaluated the association between this codon 10 polymorphism in the TGFβ1 gene and the prevalence of myopia in individuals of Chinese descent. They found an association between high myopia and the C allele. An allele is one of two forms of a gene that have the same location on a chromosome and can result in different molecular outcomes. A SNP is a change in a single nucleotide, and it is the most common type of genetic variation. For most SNPs, >1% of the human population will have it. Most SNPs are benign, but some may be associated with common diseases. In this study, the SNP rs1800470 led to a substitution of a tyrosine by a cytosine nucleotide. Thus, the allele where the SNP was present was considered the C allele (5). There was also a statistically significant difference in the prevalence of the CC genotype (when both alleles have the variant) versus the TT genotype (when neither allele has the variant) between myopic individuals and nonmyopic controls. Those with the CC genotype had the highest probability of developing high myopia.

Sandhya *et al.* (6) similarly analyzed the prevalence of the codon 10 polymorphism in myopic and nonmyopic individuals of South Indian descent and found an elevated frequency of the TC genotype in high myopia individuals as compared to controls, but the results were not statistically significant. However, they found

that low myopia groups had an elevated frequency of the CC genotype compared to controls. Sandhya *et al.* concluded that the CC genotype was associated with low myopia but not high myopia, while Lin *et al.* observed an association between the CC genotype and high myopia. One potential explanation for the opposing conclusions of these studies could be different racial and ethnic groups. We aim to discover the prevalence of this variant in the American population.

Ethnic group	Myopic	Non-myopic
European ancestry	13	7
East Asian	4	1
Asian Indian	2	0

Table 1: Major ethnic groups represented in the study and myopia status.

Researchers have recently been analyzing the associations of genes and environmental factors with myopia. Several studies have concluded that both genetic and environmental factors play a role in the development of myopia. Verhoeven *et al.* (7) found that those genetically predisposed to high myopia who also received a university-level education were much more likely to develop myopia than their similarly genetically predisposed counterparts who only received a primary level education. Wu *et al.* (6) also found a strong association between education level and the development of myopia. One hypothesis for these findings is that more time spent reading and using technology in school can impact the development of myopia due to increased eyestrain because of short-distance reading. Although several studies have analyzed how genetic and environmental factors play a combined role in myopia development, few studies have specifically evaluated the TGFβ1 codon 10 polymorphism and its interaction with the environment (8). To our knowledge, myopia and the TGFβ1 codon 10 polymorphism association studies have not been conducted before in the United States, and the TGFβ1 codon 10 polymorphism's relationship with reading and device use has yet to be evaluated.

The purpose of this study was to assess the association of this SNP (rs1800470) in the TGFβ1 gene with behavioral and demographic factors, and with the prevalence of low myopia (-0.25 to -6.00 diopters). In light of previous findings, we hypothesize that the SNP, parental diagnosis of myopia, as well as behavioral factors including increased hours spent reading and reading distances less than one foot, will be associated with an increased likelihood of developing low myopia. In this study, low myopia was evaluated because all myopic study participants only had low myopia. These findings would be helpful in behavioral modification in the prevention of myopia, considering that both environmental and genetic factors are involved in the development of myopia. In addition, knowing if a person has a genetic polymorphism that would predispose them to myopia would allow for more aggressive behavioral management to be undertaken prior to onset.

Results

Of the 300 people invited, 28 volunteers completed the survey. DNA results were obtained for 24 individuals who completed the second part of the study, which consisted of genetic testing. When analyzing the sample's demographics, 17 respondents were between the ages of 15 and 20, 7 between the ages of 30 and 49, and 4 were between the ages of 50 and 59. Twenty of the survey respondents self-identified as having European ancestry, 5 identified as East Asian, 2 as Asian Indian, and 3 as Hispanic, with 2 participants identifying with two races (**Table 1**). Twenty-two of the survey respondents were female, and 6 were male. Eighteen of the survey respondents reported that they were myopic, and 10 were non-myopic. All myopic participants had low myopia, which corresponds with either mild or moderate myopia and is indicated by an eyeglass prescription between -0.25 and -6.00 diopters.

In regard to genetic analysis, if the codon 10 SNP polymorphism in the TGFβ1 gene is present in a DNA sample, then the participant either has a TC (heterozygous, variant on one allele) or CC (homozygous, variant on both alleles) genotype. Of the 24 DNA samples that were obtained, 9 had the TT genotype (homozygous, no variant on either allele), 12 had the heterozygous TC genotype, and 3 had the homozygous CC genotype (**Table 2**).

The main statistical analysis involved risk ratio (RR) calculations, which is the quotient of the risk of an event in an exposure group and the risk of an event in a control group. Risk ratios were determined by using OpenEpi and EpiInfo (**Table 3**).

RR values were calculated according to the formula [A/(A+B)] / [C/(C+D)]. If both the upper and lower bounds of the 95% confidence interval provided by OpenEpi are greater than or below 1, then the RR value is statistically significant. The 95% CI has a 0.95 probability of containing the true RR value of the population. In using RR, less likely relationships are constrained within 0 and 1, while more likely relationships may have an RR value of 1 or greater. Thus, more likely relationships' RR

Genotype	Myopic	Non-myopic
TT	5	4
TC	9	3
CC	2	1

Table 2: Genotypic distribution with respect to myopia status.

	Self-reported as myopic	Self-reported as non-myopic
Behavior or demographic characteristic	A	B
No behavior or demographic characteristic	C	D

Table 3: Two-by-two table for calculating risk ratios.

	Myopic participants	Non-myopic participants
Neither parent myopic	5	0
One parent myopic	8	3
Both parents myopic	3	5

Table 4: Frequencies for the presence of myopia in survey respondents and their parents.

values increase faster than less likely relationships' RR values decrease.

Three RR results were obtained from the survey data with 95% confidence intervals greater than one. Participants with neither parent having myopia were 2.36 times as likely to have myopia as participants with both parents having myopia (RR 2.36; 95% CI: 1.01, 5.59). Similarly, participants with neither parent having myopia were 1.60 times as likely to have myopia as participants with at least one parent having myopia (RR 1.60; 95% CI: 1.02, 2.50) (**Table 4**).

Due to small sample size, there was no participant who reported being non-myopic in addition to having no parent with myopia, and thus a 0 was present in the calculation of these risk ratios. Therefore, as per OpenEpi guidelines, a 0.5 was used in place of the 0 value for this risk ratio calculation alone. In regards to exercise and sports, survey respondents who participated in either stationary or no physical activity at all were 1.91 times as likely to have myopia as participants who participated in in at least some non-stationary physical activity (RR 1.91; 95% CI: 1.27, 2.87).

When analyzing additional demographic and behavioral results, it was found that respondents between the ages of 15 and 20 were 1.68 times as likely to have myopia as respondents age 30 or above (RR 1.68; 95% CI: 0.84, 3.38). Male participants were 1.05 times (or almost equally) as likely to have myopia as female participants (RR 1.05; 95% CI: 0.55, 2.00). Respondents identifying as Asian Indian or East Asian were 1.32 times as likely to have myopia as respondents identifying as of European ancestry (RR 1.32; 95% CI: 0.85, 2.05). In regards to behavioral data, participants who read printed materials 1 or more hours a day were 3.70 times as likely to have myopia as participants who read printed materials for less than an hour a day (RR 3.70; 95% CI: 0.63, 21.69). Respondents who read on a device for 1 or more hours a day were 1.31 times as likely to have myopia as respondents who read on a device for less than 1 hour a day (RR 1.31; 95% CI: 0.32, 5.38). Participants who read at a distance of less than 1 foot were 1.15 times as likely to have myopia as participants who read at a distance of a foot or more (RR 1.15; 95% CI: 0.67, 2.00). These results had confidence intervals straddling 1.

The genotypic prevalence was determined using the allelic discrimination plot (**Figure 1**). The results are represented as a scatter plot of the variant allele (marked with VIC dye) versus the nonvariant allele (marked with FAM dye). For each sample in the assay, a unique pair of fluorescent dye detectors, called reporter dyes, was used. One fluorescent dye matches the C allele (VIC dye), and the other matches the T allele (FAM dye). In each sample, a probe linked with a reference dye and the reporter dye bind to the SNP site; DNA polymerase splits the probe, causing the reporter dye to fluoresce. The real-time polymerase chain reaction (PCR) instrument measures the intensity and color of the fluorescence to determine the genotype of the sample. The x and y axes indicate fluorescence intensities emitted by the VIC and FAM dyes, respectively. The subsequent allelic discrimination plot determines which alleles are in each sample (9). With the rs1800470 SNP assay, the cluster in the upper-left corner consisted of wild type individuals with no variant on either allele (TT), and the cluster in the bottom-right corner consisted of individuals with the variant on both alleles (CC). The cluster in the middle constituted heterozygous (TC) individuals. The genotype for each individual was determined using the allelic discrimination plot and the data table with Rn coordinates, with Rn signifying the ratio between fluorescence of the reporter dye and the reference dye.

The Taqman SNP genotyping assay (reference number 1800470) only considered T and C alleles. The manufacturer Thermo Fisher Scientific has shown the expected intensity range for the two alleles in a 1000 Genome allele frequency table (**Table 5**).

There were several relationships evident in the genetic data. Participants who identified as Asian Indian

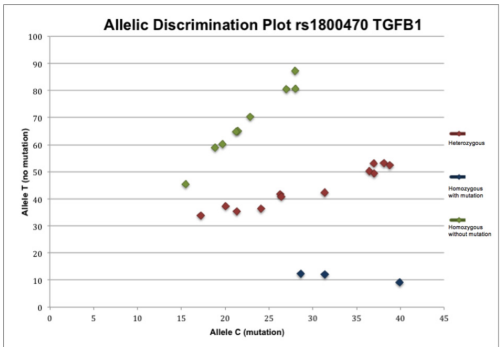

Figure 1: Allelic Discrimination Plot of the allele with no variant vs. the allele with the variant.

or East Asian were 1.30 times as likely to have the variant C allele as participants who identified as of European ancestry (RR 1.30, 95% CI: 0.70, 2.40). Participants with the single variant heterozygous genotype were 1.32 times as likely to have myopia as participants with no variant allele (RR 1.32, 95% CI: 0.68, 2.55). Participants with the TC (heterozygous) genotype were 1.13 times as likely to have mild or moderate myopia as participants with the CC (homozygous, variant on both alleles) genotype (RR 1.13, 0.47, 2.67). Participants with the CC genotype were 1.20 times as likely to have mild or moderate myopia as participants with the TT (homozygous, no variant on either allele) genotype (RR 1.20, 95% CI: 0.45, 3.23). Participants with both parents having myopia were 1.11 times as likely to have the C allele as participants with neither parent having mild or moderate myopia (RR 1.11, 95% CI: 0.45, 2.77). All myopic participants had low myopia, which is further categorized into mild (the lowest severity) and moderate myopia. In regard to the severity of myopia, participants with the single variant heterozygous genotype were 1.76 times as likely to have moderate myopia as participants with no variant allele (RR 1.76, 95% CI: 0.44, 17.07). Participants with the TC genotype were 2.78 times as likely to have moderate myopia as participants with the TT genotype (RR 2.78, 95% CI: 0.44, 17.63). Finally, participants with the CC genotype were 2.50 times as likely to have moderate myopia as participants with the TT genotype (RR 2.50, 95% CI: 0.27, 23.36). These confidence intervals straddled 1.

Discussion

In this study, the data and the RR calculations seem to support the assertion that the prevalence of the variant C allele is correlated with low myopia, although the results are not statistically significant. This may suggest that the codon 10 polymorphism is associated with myopia in accordance with the hypothesis. In consensus with previous studies (6), the CC genotype is more closely associated with low myopia than the TT. The current study also found that participants with the TC genotype were more likely to have low myopia than participants with the CC genotype, suggesting that a single variant allele is more closely associated with low myopia than two variant alleles. This is contrary to the results of Sandhya et al., which suggests that individuals with the CC genotype were more likely to have low myopia than individuals with the TC genotype. It is also possible that both conclusions are correct, with the CC genotype causing one phenotype in one race and a different phenotype in another.

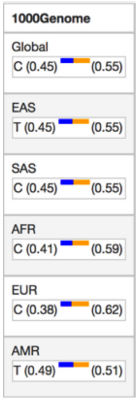

Table 5: Allele frequency table for the TGFβ1 codon 10 polymorphism, SNP ID rs1800470. The abbreviations are as follows: East Asian (EAS), South Asian (SAS), African (AFR), European (EUR) and Ad Mixed American (AMR).

All myopic participants had either mild or moderate myopia, and participants with the variant C allele were more likely to have moderate myopia than participants with no variant allele, perhaps suggesting that the presence of the SNP is associated with a higher degree of myopia. This conclusion was similar when comparing specific genotypes, with TC and CC genotypes more associated with moderate myopia than the TT genotype. Nevertheless, severity data was confounded by the fact that some myopic participants did not know their prescription, and thus could not accurately define themselves as mild or moderate. Finally, there was a higher prevalence of the variant C allele in the East Asian and Asian Indian population as compared to those of European ancestry. Racial comparisons in the prevalence of the C allele have not been conducted before as both previous myopia–TGFβ1 gene studies took place in either China or India, respectively. In regard to genetic data, this study ultimately indicated that the C allele, and in particular the TC genotype, may be associated with the prevalence of low myopia. The results further suggested that the C allele may be associated with a higher severity of low myopia and that Asian Indians and East Asians are possibly more likely to have the variant C allele than those of European ancestry; however, the RR calculations suggest that these associations are not statistically significant. One limitation of this study is the small sample size. It is understood that with a larger sample size, 95% confidence intervals will decrease and the results may be more significant.

In addition to the genetic study, several behavioral and demographic assessments were conducted. Interestingly, participants with neither parent having myopia were more likely to have myopia than participants with both parents having myopia. This result was statistically significant. Although this conclusion may seem contradictory to the partially genetic basis of myopia as established in past studies, it is speculated

that behavioral factors may explain the results. A parent with myopia may have greater awareness of the environmental factors that lead to the development of myopia, and thus may encourage their children to have healthy eye habits, including reading at a distance of more than one foot. This assertion is supported by survey data that found a positive association between a reading distance of less than a foot and myopia (RR 1.15, 95% CI: 0.67, 2.00). Future studies could examine whether respondents feel that parental vision has an influence on their own reading habits.

The relationship between specifically non-stationary exercise and myopia has not been previously studied. Another statistically significant result of this study was a positive association between non-stationary exercise and myopia: individuals engaging in no exercise were significantly more likely to have myopia compared to individuals with some level of exercise. It is hypothesized that non-stationary exercises require the eye to identify objects over medium or long distances and thus prevent the development of myopia. Nevertheless, the author acknowledges that although there is a correlation since the two factors exist together, this does not prove that non-stationary physical activity and the development of myopia are dependent upon one another or have a causal relationship.

In accordance with the hypothesis, it was also concluded that a reading distance of less than one foot and increased hours spent reading are associated with myopia. Contrary to past assertions, this study showed that participants with a high school education were more likely to have myopia than participants with a higher education. Study participants with a high school education were often less than 20 years of age while participants with a higher education were mostly older. It is speculated that the risk of myopia was higher in the younger population due to the increasingly sedentary lifestyle of young adults as well as the increased use of electronic devices. These findings could be helpful in behavioral modification in the prevention of myopia, considering that both environmental and genetic factors are involved in the development of myopia. In addition, knowing if a person has a genetic polymorphism that would predispose them to myopia, more aggressive behavioral management can be undertaken to prevent its development.

Due to limitations in the availability of adequate amounts of reagents, the SNP genotyping assay could not be replicated multiple times. However, according to information from the Department of Genome Sciences at the University of Washington, SNP genotyping assays generally have an error rate of much less than 1% (10). Additionally, data results were constrained by sample size, and the confidence intervals of several risk ratios suggest that several associations may not be significant. With more study participants, the statistical significance power of this research would be greater. To corroborate these findings, the author hopes to conduct future studies with a larger sample size. A confounding variable could be that a participant may have identified with one race/ethnicity when filling out the survey, but had other races in their ancestry. Another possible confounding variable could be that people stating that they had low myopia but not knowing their actual prescription were not able to correctly identify whether it was mild or moderate. Nevertheless, though more in-depth study is required, the study's conclusions add a unique dimension to the TGFβ1 codon 10–myopia body of knowledge.

Methods

The study was approved by the Institutional Review Board of the Walker School. Surveys were administered to juniors, seniors, and faculty at The Walker School in Marietta, Georgia. The survey requested information about age, ethnicity, reading habits, and device use, as well as myopia diagnosis, parental diagnosis of myopia, and eyeglass or contact lens prescription. The survey consisted of multiple-choice questions and one prompt asking the participant to indicate their exact eyeglass prescription. Low myopia was diagnosed as above -6.00 diopters, with mild myopia between -0.25 and -3.00D and moderate myopia between -3.25 and -6.00D. Surveys, informed consent forms, and parent signatures for those under age 18 were obtained prior to participants providing DNA samples.

A research supervisor was present at all times, including when participants arrived to provide DNA samples. Gloves, masks, and goggles were worn by the researcher throughout the process. The DNA samples and surveys were assigned a randomly generated number for identification purposes; only the research supervisor retained knowledge of a participant's true identity. Each participant rinsed with a 1% saline solution for 30 seconds and spit into a labeled tube that was subsequently centrifuged for 10 minutes. Once a solid DNA pellet appeared at the bottom of the tube, the clear liquid suspended above it was poured out of the tube. In order to resuspend cells, a micropipet was used to transfer 50 µl of cell pellet to a screw-cap microtube containing 200 µl of Chelex. The labeled tube was placed in a boiling water bath for 10 minutes. Afterwards, the tube was vigorously shaken for 5 seconds and then centrifuged for 90 seconds. 50 µl of the DNA supernatant was transferred into a clean, labeled microtube. The microtubes were then placed in the freezer in anticipation of the SNP assay process.

Initially, PCR amplification, restriction enzyme digestion, and gel electrophoresis were conducted

in order to obtain genotype results, according to the procedure outlined in a published study (11). However, time constraints limited efforts to optimize the PCR process. Ultimately, a Thermo Fisher TaqMan SNP Genotyping Assay for SNP ID rs1800470 was utilized in order to obtain genotype results.

The standard protocol in the Thermo Fisher TaqMan SNP Genotyping Assay User Guide was followed with a few minor alterations (12). The reaction mix was prepared for the wet DNA method. A 96-well plate was utilized with a 25 µl reaction in each well. Each reaction contained 12.5 µl of OneTaq Hot Start 2X Master Mix with Standard Buffer (New England BioLabs), 1.25 µl of the SNP Genotyping Assay 20X Working Stock (Thermo Fisher Scientific), 4.0 µl of DNA supernatant, and 7.25 µl of nuclease-free water. After all components were added to each well, the plate was covered with MicroAmp Optical Adhesive Film and then centrifuged to spin down the contents and eliminate air bubbles.

The reaction plate subsequently underwent real-time PCR using the LightCycler 480 System in the Standard mode thermal cycling setting. That is, thermal cycle parameters included AmpliTaq Gold® activation at 95°C for 10 minutes for one cycle, repetitive denaturation at 95°C for 15 seconds, and annealing at 60°C for one minute for 40 cycles. The reaction volume was 25.0 µl. After PCR amplification, a post-PCR plate read was performed on the real-time PCR instrument. The real-time PCR instrument measures fluorescence and then plots R_n values based on the fluorescence signals for each well. The R_n value is the ratio between the fluorescence of the marker dye, which is attached to either the variant or nonvariant allele, and the reference dye. Statistical analysis of the survey and genomic data was performed using Excel, Epi Info, and OpenEpi to calculate prevalence and risk ratios (RR) (13).

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Acknowledgments

Research was conducted between August 2015 and January 2016 at The Walker School and at the Hudson Lab, Kennesaw State University. Funding for this project was provided by The Walker School. The OneTaq Master Mix and restriction enzyme (with buffer) were donated by New England BioLabs.