Association of Matrix Metalloproteinase Gene Polymorphisms with Refractive Error in Amish and Ashkenazi Families

Robert Wojciechowski,[1] *Joan E. Bailey-Wilson,*[1] *and Dwight Stambolian*[2,3]

Purpose. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are involved in scleral extracellular matrix remodeling and have shown differential expression in experimental myopia. The genetic association of refractive error and polymorphisms in MMP and TIMP genes in Old Order Amish (AMISH) and Ashkenazi Jewish (ASHK) families was investigated.

Methods. Individuals from 55 AMISH and 63 ASHK families participated in the study. Ascertainment was designed to enrich the families for myopia; the mean spherical equivalent (MSE) refractive error (SD) was −1.61 (2.72) D in the AMISH, and −3.56 (3.32) D in the ASHK. One hundred forty-six common haplotype tagging SNPs covering 14 MMP and 4 TIMP genes were genotyped in 358 AMISH and 535 ASHK participants. Association analyses of MSE and the spherical component of refraction (SPH) were performed separately for the AMISH and the ASHK. Bonferroni-corrected significance thresholds and local false discovery rates were used to account for multiple testing.

Results. After they were filtered for quality-control, 127 SNPs were included in the analyses. No polymorphisms showed statistically significant association to refraction in the ASHK (minimum $P = 0.0132$). In AMISH, two SNPs showed evidence of association with refractive phenotypes: rs1939008 ($P = 0.00016$ for SPH); and rs9928731 ($P = 0.00026$ for SPH). These markers were each estimated to explain <5% of the variance of SPH in the AMISH sample.

Conclusions. Statistically significant genetic associations of ocular refraction to polymorphisms near *MMP1* and within *MMP2* were identified in the AMISH but not among the ASHK families. The results suggest that the *MMP1* and *MMP2* genes are involved in refractive variation in the AMISH. Genetic and/or environmental heterogeneity most likely contribute to differences in association results between ethnic groups. (*Invest Ophthalmol Vis Sci.* 2010;51:4989–4995) DOI:10.1167/iovs.10-5474

From the [1]Inherited Disease Research Branch, National Human Genome Research Institute, National Institutes of Health (NIH), Baltimore, Maryland; and the Departments of [2]Ophthalmology and [3]Genetics, University of Pennsylvania, Philadelphia, Pennsylvania.

Supported by U.S. Public Health National Eye Institute Grant EY020483 (DS) and in part by funds from the intramural program of the National Human Genome Research Institute, NIH (RW, JEB-W). Genotyping services were provided by the Center for Inherited Disease Research (CIDR) which is fully funded through Federal Contract N01-HG-65403 from the NIH to Johns Hopkins University.

Submitted for publication March 4, 2010; revised April 5, 2010; accepted April 23, 2010.

Disclosure: **R. Wojciechowski**, None; **J.E. Bailey-Wilson**, None; **D. Stambolian**, None

Corresponding author: Robert Wojciechowski, National Human Genome Research Institute, National Institutes of Health, 333 Cassell Drive, Suite 1200, Baltimore, MD 21224; robwoj@mail.nih.gov.

Clear vision requires that images formed by incident light are focused at the eye's retinal plane. The maintenance of clear, focused, vision (i.e., emmetropia) through adulthood depends on a precise and complex regulation of eye shape during ocular development. This regulation ensures that the eye's focal and anatomic lengths are aligned. Any discrepancy between the position of the retina and the eye's focal point results in the spherical refractive errors myopia and hyperopia. In myopia, images of distant objects formed by the eye's optical system focus anterior to the retinal plane, causing blurred distance vision. In its functional opposite, hyperopia, these images focus behind the retina (in the unaccommodating eye). Hyperopia is associated with blurred near vision. Myopia and hyperopia are fundamentally qualitative definitions. However, the degree of departure from perfect focus can be quantified as the refractive error. Typically, refractive error represents the dioptric power of optical lenses necessary to achieve proper distance correction. By convention, negative values of refractive error (in diopters, D) represent myopia, whereas positive values represent hyperopia.

Although there is a broad scientific consensus that environmental and behavioral factors influence refractive development and the genesis of myopia, genetic epidemiologic studies have consistently shown that the distribution of ocular refraction within populations is largely determined by genetics. Heritability estimates for refraction are usually very high across a wide spectrum of ethnic groups despite various intraethnic prevalences of myopia and myopigenic environmental influences. For example, we reported the heritability of refractive error in an adult Old Order Amish (AMISH) population to be 70%.[1] High heritabilities are remarkably consistent across studies with estimates reported for Caucasian Americans,[1–3] Europeans,[4–7] and African Americans,[3] ranging between 60% and 90%.

Structurally, myopia is caused by an excessive axial length of the eye relative to its optical power.[8] Hence, myopic eyes tend to be longer than nonmyopic eyes.[8] The converse is true of hyperopia (i.e., hyperopic eyes are comparatively shorter than emmetropic and myopic eyes). Anatomically, these variations in axial length are due primarily to differences within the posterior (or vitreous) chamber, which occupies the bulk of the eye's volume. Although the molecular signal for blur-induced differential eye growth is thought to originate from within the retina, ocular elongation must occur via the growth of the outer tunic of the eye: the sclera. In mammals, the sclera is a fibrous connective tissue composed mainly of extracellular collagen, which accounts for up to 90% of the sclera's dry weight (Zorn N, et al. *IOVS* 1992;33:ARVO Abstract 1811). The majority (i.e., up to 99%) of scleral collagen is of the type I variety, although low levels of other collagen subtypes have also been found (Norton TT, et al. *IOVS* 1995;36:ARVO Abstract 3517). The structural organization of the scleral extracellular matrix (ECM) depends largely on the cellular activity of fibroblasts, the main ECM-producing cells in the sclera. The degradation of the ECM that occurs during scleral

remodeling in eye growth and myopization (i.e., myopia development) is partially regulated through members of a major family of zinc- and calcium-dependent endopeptidases: the matrix metalloproteinases (MMPs) (see Rada et al.[9] for a review). In humans, the MMP gene family comprises 23 distinct genes distributed across the genome; 9 MMP genes are located in a cluster at 11q22.2. The MMPs play numerous important roles in regulating cell–matrix composition in connective tissue and have been implicated in normal developmental processes and the pathogenesis of a variety of diseases.[10,11] The expression of MMPs is generally low in tissues and is induced during active ECM remodeling.[12] MMP regulation is mostly achieved at the transcriptional level but can occur at multiple stages. Activation of latent zymogens must occur for the MMPs to gain their proteolytic activity. Once activated, MMPs can be inactivated by multiple mechanisms, including direct interactions with one of four tissue inhibitors of matrix metalloproteinases (TIMPs).[12]

Given the important role of MMPs in ECM composition and remodeling, genetic variations in MMP genes are potential candidates for refractive error heritability and susceptibility to myopia. Indeed, a recent candidate gene study of older British adults reported statistically significant genetic associations between *MMP3* and *MMP9* polymorphisms and common myopia.[13] We hypothesize that genetic polymorphisms in MMP and/or TIMP genes contribute to intrapopulation variations of refractive error measured as a quantitative trait. In the present study, we conducted family-based association analyses of genetic polymorphisms within MMP and TIMP candidate regions that included: *MMP1*, *-2*, *-3*, *-7*, *-8*, *-9*, *-10*, *-12*, *-13*, *-14*, *-20*, and *-27*, as well as *TIMP1*, *-2*, *-3*, and *-4*. These candidate regions were chosen to include genes whose homologs have shown differential expression in animal myopia models,[14–17] as well as polymorphisms within a cluster of nine MMP genes situated at 11q22.2. Analyses were conducted on a subset of individuals from 63 Orthodox Ashkenazi Jewish (ASHK) and 55 Old Order Amish (AMISH) American families participating in the Myopia Family Study. We found statistically significant genetic associations between two single-nucleotide polymorphisms (SNPs), rs1939008 and rs9928731, and refractive error in the AMISH, but not the ASHK; rs1939008 is located between *MMP1* and *MMP10*, and rs9928731 is located within *MMP2*.

Materials and Methods

Participants

All study participants were recruited to participate in the Myopia Family Study (MFS), a multiethnic, family-based genetic linkage study of myopia. MFS recruitment strategies and criteria are discussed in detail elsewhere and are briefly described here.[18–20] Families were recruited from ASHK and AMISH communities around Lakewood, New Jersey, and Lancaster County, Pennsylvania, respectively. Initially, community liaisons were used to identify myopic probands. All identified myopic probands and their families were invited to participate in the study. A nuclear family was eligible to participate in the genetic linkage study if it contained at least one pair of myopic relatives, the parents were discordant for myopia, and biological samples were available for at least two myopic individuals. This recruitment strategy was designed to select multiplex myopic families and augment the sample for families with autosomal dominantly transmitted myopia. Larger pedigrees were then formed by extending nuclear families through first- and second-degree relatives and by merging related nuclear families. Individuals were excluded from the study if they had a history of ocular or systemic diseases that could cause refractive error or affect the precision of refractive measurements. These exclusions included a history of prematurity, poorly controlled diabetes mellitus, connective tissue disorders, corneal opacities, keratoconus, retinal dystrophies and degenerations, and ocular syndromes in which myopia is a common feature. All participants provided informed consent (or assent), and the study protocol adhered to the tenets of the Declaration of Helsinki. The study was approved by the review boards of the University of Pennsylvania and the National Human Genome Research Institute.

The study sample consisted of a subset of 63 ASHK and 55 AMISH families from the MFS. The pedigrees comprised 1189 ASHK and 657 AMISH individuals. Of these, 535 (45%) ASHK and 358 (55%) AMISH participants provided DNA for genotyping. The mean pedigree size was 18.8 (8.5 genotyped) ASHK members and 11.9 (6.5 genotyped) AMISH. Extended pedigrees ranged from 4 to 85 individuals. Sample pedigree characteristics are summarized in Table 1.

Phenotyping

Study participants underwent a comprehensive eye examination that included medical and ocular health history, visual acuity, slit lamp biomicroscopy, Goldmann applanation tonometry, funduscopy, automated refractometry, and manifest refraction. Cycloplegic refraction using 0.5% cyclopentolate or 1% tropicamide was performed on all subjects younger than 41 years of age. When participants could not be examined in our study clinics, refraction data were obtained from their eye care providers.

The cycloplegic refraction providing the best visual acuity was used to define the quantitative phenotype for subjects less than 41 years of age. For older subjects and participants for whom cycloplegic refraction was unavailable, manifest refraction was used. Both mean spherical equivalent refractive error (MSE, in diopters) and the mean spherical component (SPH) were used in the analyses. MSE is obtained by adding the spherical component of the refraction to one-half of the cylindrical component, and then averaging between the eyes; SPH was defined as the mean spherical component when the refraction is measured in negative cylinders.

Marker Selection

Eight candidate regions were identified from the locations of 12 MMP and 4 TIMP genes. These included genes whose homologs have shown differential expression in experimental myopia models (*MMP14*, *MMP1*, *MMP2*, *TIMP2*, and *TIMP3*).[14–17] In addition, *TIMP1*, *TIMP4*, and *MMP9* and a ~428-kb region on chr 11 which contains a cluster of nine MMP genes (*MMP1*, *-2*, *-3*, *-7*, *-8*, *-10*, *-13*, *-20*, and *-27*) were interrogated. The characteristics of candidate regions are shown in Table 2.

SNPs were selected a priori to provide adequate coverage for common genetic variants within candidate regions. Specifically, haplotype tagging SNPs from the CEU population of the Human HapMap project (public release 23; www/hapmap.org) were chosen to cover all common SNPs (MAF $\geq$ 0.15) within candidate regions at a pairwise $r^2 \geq 0.7$. In addition, all known nonsynonymous coding SNPs with a MAF $\geq$ 0.05 in Caucasian populations were selected from the dbSNP database (http://www.ncbi.nlm.nih.gov/snp; provided in the public

Table 1. Study Sample Characteristics

Population	Families	*n**	M (%)	F (%)	Genotyped (%)	Age (SD)	MSE (SD)
AMISH	55	657	303 (46)	354 (54)	358 (55)	36.7 (17)	−1.61 (2.72)
ASHK	63	1189	615 (52)	574 (48)	535 (45)	39.6 (19)	−3.56 (3.32)
Total	118	1846	918 (50)	928 (50)	893 (48)		

* Total number of individuals in the pedigrees.

TABLE 2. Candidate Gene and Region Characteristics

Cytogenetic Location	Physical Location (kbp)*	Region Width (kb)	Local Gene Symbols	SNPs (*n*)	SNP Density (kb/SNP)	Nonsynonymous SNPs (*n*)
3p25.2	12,195–12,201	6	*TIMP4*	2	3.0	0
11q22.2	102,398–102,826	428	*MMP7; MMP20; MMP27; MMP8; MMP10; MMP1; MMP3; MMP12; MMP13*	101	4.2	11
14q11.2	23,306–23,317	11	*MMP14*	6	1.8	1
16q12.2	55,513–55,541	28	*MMP2*†	6	4.7	1
17q25.3	76,849–76,918	69	*TIMP2*	11	6.3	0
20q13.12	44,637–44,645	8	*MMP9*	6	1.3	1
22q12.3	33,197–33,259	62	*TIMP3*	12	5.2	0
Xp11.23	47,442–47,445	3	*TIMP1*	2	1.5	0
Total		615		146	4.2	14

* Human GRCh37/hg19 Assembly, February 2009.
† *MMP2* has multiple documented isoforms. The physical location and region width are for isoform a.

domain by the National Center for Biotechnology Information, Bethesda, MD) for genotyping. For the purpose of analysis, genetic locations were specified in centimorgans (cM) and extracted from the combined Rutgers linkage map.[21]

DNA Extraction and Genotyping

Venipuncture was used to collect peripheral blood from participating family members. High-molecular-weight genomic DNA was extracted from the blood samples with a DNA purification kit (Puregene; Gentra Systems, Inc; Minneapolis, MN). The purified DNA was then stored in a refrigerated DNA repository under a unique sample code. Custom SNP genotyping was performed at the Center for Inherited Disease Research (Johns Hopkins Medical Institutions, Baltimore, MD) on a bead array genotyping system (BeadLab system with GoldenGate chemistry; Illumina, Inc., San Diego, CA).

Quality Control and Data Cleaning

A total of 146 SNPs were genotyped in the MMP and TIMP candidate regions. Of these, 19 (13%) were excluded from analyses because they failed our quality criteria: 8 were excluded because of call rates below 95% and 11 because of atypical raw-intensity clustering patterns. All the remaining SNPs satisfied Hardy-Weinberg proportion expectations in ASHK founders and were included in the analyses. Two SNPs did not satisfy Hardy-Weinberg proportions in AMISH founders and were excluded from the analyses. Hence, 127 high-quality SNPs were used in genetic association analyses of ASHK, and 125 in AMISH families.

Six ASHK and 22 AMISH samples were not released due to significantly poor genotyping performance (call rate, <0.95). The remaining samples were checked for Mendelian transmission inconsistencies using the statistical programs Pedcheck[22] and Pedstats[23]; the Relcheck[24] software package was used to confirm putative pedigree relationships. All pedigrees were free of Mendelian inconsistencies, and all remaining individuals were therefore used in the analyses. Large pedigrees were split to accommodate the default memory limits of multipoint analysis in the program Merlin[25] (i.e., 24 bits for the Lander-Green algorithm), making sure that no individuals were duplicated across pedigrees.

Statistical Analysis

All analyses were conducted separately in the ASHK and AMISH families. Quantitative trait genetic association analyses were performed with the maximum-likelihood, family-based method implemented in the Merlin package.[26] The method employs a linear regression framework that models the expected phenotypic value as a function of the additive genotypic effect at each marker. To allow for correlations between related individuals, the variance-covariance matrix is modeled as a combination of genetic and environmental components. Familial relationships are taken into account by specifying marker-specific, identity-by-descent estimates and intrafamilial kinship coefficients in the covariance matrix. The approach also incorporates an imputation step in which the expected genotype scores (i.e., the expected number of minor alleles) are calculated for missing markers and included in the likelihood computations.

Statistical Significance

A family-wise type-I error rate (FWER) of $P < 0.05$ was adopted as the threshold for statistical significance. Because testing was conducted at multiple markers, the statistical significance criterion was adjusted by Bonferroni correction by dividing the FWER by the number of independent haplotype blocks in the ASHK sample (at an r^2 threshold of 0.8 using the confidence intervals method of Gabriel et al.[27] (implemented in the program Haploview, ver. 4.1[28]). The AMISH sample contained fewer haplotype blocks and the more stringent correction using the ASHK was therefore used for both populations. Hence, a pointwise $P < 4.95 \times 10\text{E-}4$ (0.05/101 haplotype blocks) was considered statistically significant at a FWER < 0.05.

In addition to pointwise *P* values, we computed *q* values, which are related to the false-discovery rate[29,30] (FDR, the expected proportion of false-positive findings among all rejected null hypotheses). The *P*-value of a statistic s_i gives the probability of having a statistic more extreme than s_i under the null hypothesis. The *q*-value associated with s_i is defined as the minimum FDR level at which s_i would be judged significant. Given that the *q*-value depends on the distribution of ordered test statistics in a given experiment, it automatically accounts for multiple testing and is useful in multiple-hypothesis association analyses. A population-specific $q \leq 0.05$ was considered statistically significant in the present study.

RESULTS

For the ASHK families, the maximum association signal ($P = 0.0132$ for MSE; $P = 0.0136$ for SPH) was obtained for rs11225314, which is located between MMP7 and MMP20 on chromosome 11. However, this marker did not meet our Bonferroni-corrected statistical significance threshold of $P < 4.95 \times 10\text{E-}4$. Moreover, no marker showed a nominal $P < 0.05$ in both the ASHK and AMISH families.

In analyses of AMISH families, rs1939008 ($P = 0.00,016$) and rs9928731 ($P = 0.00026$) reached statistical significance for SPH. Analyses of MSE in AMISH yielded statistically significant association for rs9928731 ($P = 0.00043$) and borderline significance for rs1939008 ($P = 0.00084$). The corresponding *q* values associated with rs1939008 and rs9928731 were $q = 0.045$ in the MSE analysis, and $q = 0.013$ in the SPH analysis. Marker rs1939008 is located on chromosome 11 approximately 4.2 kb downstream of *MMP1* and 5.1 kb upstream of the start of *MMP10* (Human GRCh37/ hg19 Assembly). Marker rs9928731 is located on chr 16 in an intronic region of MMP2 between coding exons 6 and 7. Table 3 summarizes association results for the most significant markers in the analyses.

Discussion

We found two SNPs within MMP and TIMP candidate regions that were significantly associated with measures of ocular refraction in AMISH families but not in ASHK Jewish families. The strongest and most consistent association signal came from rs9928731 which is located between the sixth and seventh exons of *MMP2*; the second statistically significant signal for SPH, at rs1939008, is located between *MMP1* and *MMP10*. *Q* values ($q = 0.013$ for SPH; $q = 0.045$ for MSE) for both markers were below our local FDR significance threshold of $q = 0.05$.

Although polymorphisms within or adjacent to *MMP2*, *MMP1*, and *MMP10* appear to be associated with ocular refraction in the AMISH sample, they showed no significant association in the ASHK group. Several possibilities could explain this discrepancy. First, the SNPs showing the highest association signals may not be the causative polymorphisms for ocular refractive development. The true causative alleles are more likely to be in linkage disequilibrium (LD) with rs9928731 and rs1939008. We believe, however, that better tagging of untyped, true causal alleles in the AMISH compared with the ASHK is an unlikely source of the difference in our findings between the groups. Specifically, the LD pattern surrounding rs1939008 on chr 11 appears virtually identical in the AMISH and ASHK samples (Fig. 1), although the ASHK samples showed slightly less LD than the AMISH around rs9928731 (MMP2 on chr 16). Moreover, allele frequencies of typed SNPs were similar between the study populations (Table 3). Hence, the putative true causative polymorphisms would have been equally well tagged by the selected markers in both the AMISH and ASHK groups. It is therefore unlikely that differences in LD structure contributed significantly to the discrepancy in our association findings. Second, both sets of families were sampled from largely endogamous, rapidly expanding, populations. It is possible that causative polymorphisms were introduced into the AMISH founder population after phylogenetic divergence from a common ancestral population. We believe this scenario to be possible but not very likely, considering the similarities in allele frequencies and LD patterns between the ASHK and AMISH within the regions of interest (Table 3, Fig. 1). Third, the distribution of ocular refraction in the underlying AMISH and ASHK populations is drastically different. Orthodox Jewish populations have been shown to have high rates of myopia, especially among males.[31,32] This is thought to be due to the influence of behavioral and environmental factors favorable for myopization, such as a strong community emphasis on lifelong religious scholarship, frequent prayer, and prolonged reading and devotional study. On the other hand, relatively low prevalences of refractive errors[1] have been reported among the Old Order Amish, who live rural agrarian lifestyles, oppose any forms of higher education, and eschew modern technological conveniences such as computers and televisions. Hence, the more extreme environmental factors that are ubiquitous in ASHK society may have introduced additional environmental variance in the ASHK cohort relative to the AMISH, potentially masking the genetic signal. This possibility would lead to lower power to detect association in the ASHK group, as the proportion of the variance due to additive genetic effects (i.e., the broad-sense heritability) would have been correspondingly smaller. It is also possible that the strong myopia-inducing environment among the ASHK overrides the heritable effect of polymorphisms in MMP genes on the distribution of refractive error. If this is the case, we would not expect replication of our findings in populations, such as South Asian Chinese and Japanese, with high prevalences of environmentally induced myopia.

Despite the relatively low prevalence of both myopia and hyperopia among the AMISH, the heritability of refractive error has been estimated to be 70% in this population.[1] This high heritability suggests that genetic factors account for a signifi-

Table 3. Association Results of the Most Significant SNPs ($P \leq 0.05$) with MSE and Mean SPH of Refractive Error

					Allele Frequencies and *P*-values					
					ASHK			AMISH		
Marker	Chr	Position (bp)	Local Genes	Function	Freq.	MSE *P*	SPH *P*	Freq.	MSE *P*	SPH *P*
rs4754850	11	101,914,290	*MMP7; MMP20*	Intergenic	0.199	0.04	0.037	0.125	0.105	0.119
rs938595	11	101,921,069	*MMP7; MMP20*	Intergenic	0.486	0.26	0.25	0.35	0.032	0.0083
rs11225314	11	101,923,574	*MMP7; MMP20*	Intergenic	0.104	0.0132	0.0136	0.083	0.0098	0.0102
rs17174327	11	101,983,656	*MMP20*	Intron	0.113	0.042	0.04	0.161	0.7	0.99
rs11225388	11	102,080,949	*MMP27*	Intron	0.205	0.64	0.61	0.302	0.084	0.038
rs12361599	11	102,083,759	*MMP27; MMP8*	Intergenic	0.205	0.64	0.61	0.302	0.084	0.039
rs2012390	11	102,095,987	*MMP8*	Intron	0.263	0.58	0.56	0.19	0.023	0.02
rs11225394	11	102,100,623	*MMP8*	Intron	0.205	0.59	0.55	0.298	0.104	0.049
rs11225400	11	102,107,932	*MMP8; MMP10*	Intergenic	0.205	0.59	0.54	0.298	0.104	0.049
rs10791591	11	102,110,789	*MMP8; MMP10*	Intergenic	0.37	0.7	0.65	0.421	0.054	0.0112
rs10791595	11	102,140,711	*LOC100128088*	Intron	0.249	0.7	0.62	0.393	0.027	0.0125
rs4754880	11	102,160,697	*MMP10; MMP1*	Intergenic	0.124	0.99	0.9	0.165	0.048	0.0138
rs1939008*	11	102,161,633	*MMP10; MMP1*	Intergenic	0.188	0.89	0.95	0.256	0.00084	0.00016†
rs2239008	11	102,166,290	*MMP1*	UTR; 3	0.124	1	0.9	0.165	0.048	0.0138
rs7125062	11	102,168,713	*MMP1*	Intron	0.217	0.34	0.44	0.215	0.11	0.049
rs655044	11	102,329,270	*MMP13*	Intron	0.127	0.025	0.03	0.219	0.28	0.23
rs17242319	16	54,077,035	*MMP2*	Coding; synonymous	0.292	0.5	0.48	0.417	0.025	0.0094
rs9928731*	16	54,080,512	*MMP2*	Intron	0.405	0.18	0.22	0.5	0.00043†	0.00026†
rs243845	16	54,083,988	*MMP2*	Intron	0.471	0.67	0.7	0.331	0.035	0.054
rs11639960	16	54,090,771	*MMP2*	Intron	0.301	0.72	0.66	0.417	0.023	0.0087
rs2236416	20	44,073,982	*MMP9*	Intron	0.115	0.96	0.95	0.041	0.023	0.022
rs2274756	20	44,076,517	*MMP9*	Missense	0.119	0.97	0.94	0.041	0.023	0.022

Freq., frequency of reference alleles in the AMISH and ASHK populations; minor alleles were the same for all SNPs in both subgroups.

* Q values for rs1939008 and rs9928731 were 0.045 and 0.013 for the MSE and SPH analyses, respectively.

† Statistically significant at $P < 0.000495$.

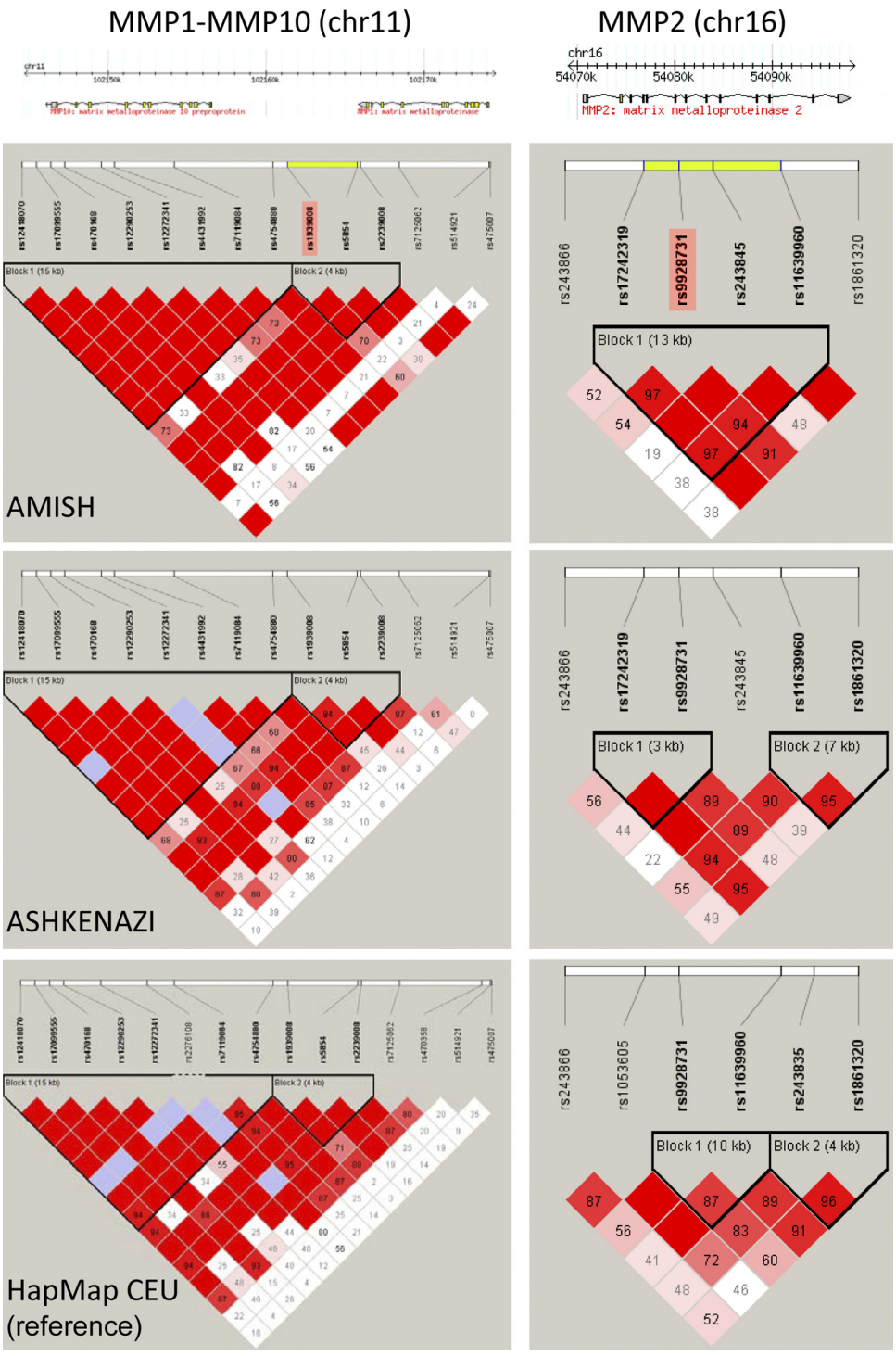

FIGURE 1. Linkage disequilibrium (LD) patterns in areas adjacent to rs1939008 (chr 11, *left*) and rs9928731 (chr16, *right*) for the AMISH (*top*), ASHK (*middle*), and HapMap CEU reference population (*bottom*) samples. CEU LD structures are provided for comparison purposes. Statistically significant SNPs are highlighted in *red*. Haplotype blocks were defined separately in independent ASHK and AMISH trios and founders by using the confidence intervals method of Gabriel et al.[27] at an r^2 threshold of 0.8. Linkage disequilibrium was calculated and images were exported from the Haploview program,[28] version 4.1 (http://www.broadinstitute.org/haploview). *Red boxes*: complete pairwise LD ($D' = 1$) at an LOD ≥ 2. *Blue boxes*: complete pairwise LD at lower confidence levels (LOD < 2). Shades of *red* show pairwise $D' < 1$ at an LOD ≥ 2. *White boxes*: $D' < 1$ and at LOD < 2. *Numbers* within boxes show the pairwise D' estimates ($\times 100$). *Empty boxes* represent $D' = 1$.

cant proportion of the population variance of ocular refraction in the AMISH. Nevertheless, the proportion of the total phenotypic variance of SPH in the AMISH sample explained by rs9928731 and rs1939008 in the present study was estimated to be 4.34% and 4.84%, respectively, under a general additive model. This finding suggests that a large proportion of the genetic heritability of refractive error is due to genes other than *MMP2*, *MMP1*, and/or *MMP10*. It should be noted, however, that these marker-specific heritability estimates are likely to differ from the true heritability in the AMISH population from which the families were recruited. Specifically, the sampled AMISH families were enriched for myopia and were thus not phenotypically representative of the overall community. Moreover, rs9928731 and rs1939008 are most likely not the true causative polymorphisms responsible for refractive development but in LD with the biologically relevant genetic variants. Marker-specific heritability estimates in our analyses should therefore be interpreted with caution.

The regulation of cell–extracellular matrix composition is highly dependent on MMPs and their interactions with a number of substrates. Although the expression patterns of MMPs are tissue- and cell type-specific, microarray gene expression data from Seko et al.[33] (GEO accession GSM277264; http://www.ncbi.nlm.nih.gov/geo/ provided in the public domain by

NCBI, Bethesda, MD) show that *MMP1* and *MMP2* (though not *MMP10*) RNA are expressed in cultured human infant scleral cells. Moreover, differential MMP2 expression has been implicated in scleral remodeling in experimental myopia studies in tree shrews[14,16,17,34] and chicks.[15,35] In these form-deprivation animal models, myopic eyes show increased *MMP2* mRNA expression compared with that in normally developing eyes, leading to increased collagen degradation and active scleral remodeling. A similar mechanism may be involved in common forms of heritable human refractive error. However, the role of *MMP2* in the control of refractive development requires further study and refinement in both animal models and human genetic epidemiologic studies.

To our knowledge, only two studies have been conducted to investigate the association of MMP polymorphisms and refractive error phenotypes.[13,36] Liang et al.[36] studied the effects of 13 polymorphisms in *MMP3* and *TIMP1* on the risk of high myopia among Taiwanese individuals.[36] They did not find statistically significant associations with these SNPs in the full analysis of 216 cases and 474 controls. However, a subset analysis of highly educated subjects yielded a positive association with a polymorphism in *MMP3*, suggesting a possible gene–environment interaction effect on high myopia. In a study of older British adults, Hall et al.[13] reported increased risks of common myopia (spherical equivalent refractive error ≤ -1 D) among carriers of promoter polymorphism alleles known to increase the expression of *MMP3* and *MMP9*.[13] They also observed a significant trend of increasing risk of myopia with the combined dosage of the transcription enhancing alleles of *MMP1*, *MMP3*, and *MMP9* promoter polymorphisms. Although they did not test markers in other MMP regions, their combined evidence suggests that increased MMP transcription is associated with higher risk of myopia within the nonpathologic ranges.

In the present study, we did not confirm the findings of Hall et al.[13] of associations with *MMP3* and *MMP9* polymorphisms in either the AMISH or ASHK families. This inconsistency may be due to several factors, including demographic and population genetic differences between the studies; incomplete coverage of our custom SNP panel; discrepancies in sampling methodologies; differences in the respective phenotype coding schemes (i.e., quantitative versus binary trait coding); limited statistical power; and type-1 error. Nevertheless, common mechanisms may underlie refractive modulation by both *MMP2* polymorphisms found in the AMISH families and *MMP9* variants.[13] Specifically, both *MMP2* (gelatinase-A) and *MMP9* (gelatinase-B) are capable of degrading common collagenous and noncollagenous substrates. Moreover, inactive MMP2 precursor can be activated into its proteolytically active form by active MMP9, and both MMP2 and MMP9 are capable of cleaving latent transforming growth factor (TGF)-β cytokines, leading to ECM remodeling.[37,38] These biochemical properties are significant in human refractive error, because TGF-β1 and -β2 polymorphisms have been associated with high myopia in two separate studies of Taiwanese subjects.[39,40] Hence, the similar properties of the gelatinases, as well as the mechanisms of MMP2 activation by MMP9, and TGF-β activation by both MMP2 and MMP9 provide common pathways by which *MMP2* and *MMP9* variants could influence scleral remodeling and refractive modulation.

Our results in AMISH families also support a potential role for *MMP1* or *MMP10* variants in refractive error heritability. The human *MMP1* and *MMP10* genes code for collagenase-1 and stromelysin-2 preproteins, respectively, and are located in the chromosome 11q22.2 MMP gene cluster. To our knowledge, MMP10 has not been shown to be expressed in mammalian sclera, although it is synthesized in mouse choroid.[41] MMP1 proteins, however, are abundant in normal eye tissues,[42] particularly in the ciliary muscle, iris, corneal endothelium, ciliary nonpigmented epithelium and anterior sclera, where they are thought to regulate aqueous humor outflow. Their role in myopization and refractive development, however, remains unclear. The *MMP1* secreted enzyme (collagenase-1) breaks down type I and III interstitial collagens, which are both found in the human sclera.[43,44] MMP1 can be activated to degrade collagen by the plasmin–plasminogen activator system.[45] Functional *MMP1* polymorphisms could therefore affect the breakdown of scleral type I and/or type III collagen, either directly or via differential molecular interactions with plasmin. These *MMP1*-mediated pathways for tissue remodeling, however, have not been investigated as mechanisms for myopization. Hence, more study is necessary to determine the role of *MMP1* and *MMP10* in normal ocular physiology and refractive error control.

MMP1 and *MMP2* may also affect eye growth through interactions with insulin-like growth factor binding proteins (IGFBPs) and insulin-like growth factors I and II (IGF-I and IGF-II). IGFs are fundamental cell regulators involved in cell adhesion and migration, and the regulation of the cell cycle and apoptosis (see Firth and Baxter[46] for a review). A recent study in an avian myopia model showed that IGF-I is a powerful stimulator of myopic ocular elongation in response to optical defocus.[47] The availability of IGFs to target IGF receptors is believed to be regulated by IGFBP binding.[48] The IGFBP 3 isoform, which binds most IGF in the body, can be cleaved by *MMP1* and *MMP2*, releasing IGFs and stimulating cell proliferation by these proteins. Hence, *MMP1* and *MMP2* regulation of IGF activity through IGFBP 3 proteolysis offers another molecular pathway through which polymorphisms in these genes may influence refractive development.

We have proposed several pathways through which polymorphisms in *MMP1* and *MMP2* could affect human refractive variation. However, specific experimental and epidemiologic data supporting any of these mechanisms are sparse. Moreover, epidemiologic evidence suggests that between-population differences in refractive error distribution are, in part, accounted for by environmental and behavioral factors. We postulate that discrepancies in genetic association results between the AMISH and ASHK in the present study may be the result of interactions between *MMP1* (or *MMP10*) and *MMP2* variations and unmeasured environmental risk factors. Taken together, results from the current investigation and previous studies illustrate the complexity of the mechanisms and multiple interacting networks involved in refractive error regulation in human populations.

Conclusion

We conducted a family-based, candidate-pathway association analysis of MMP and TIMP polymorphisms with ocular refraction in ASHK and AMISH families. Two SNPs (rs1939008 and rs9928731) were significantly associated with refraction in the AMISH, but not in the ASHK, families. Our results implicate *MMP2* and *MMP1* (and/or *MMP10*) as regulators of nonpathologic refractive error. Moreover, genetic heterogeneity or gene–environment interactions may explain interpopulation differences in the causes of refractive error variation.

Acknowledgments

The authors thank the families for their participation in the study; Reuvain Shanik, MD, and Rabbi Yitzchok Rozsansky for enthusiastic support of the Myopia Family Study; the staff of the Amish Eye Clinic in Strasburg, PA; and local optometrists and ophthalmologists for their invaluable aid in participant recruitment.

References

1. Peet JA, Cotch MF, Wojciechowski R, Bailey-Wilson JE, Stambolian D. Heritability and familial aggregation of refractive error in the Old Order Amish. *Invest Ophthalmol Vis Sci.* 2007;48(9):4002–4006.
2. Klein AP, Suktitipat B, Duggal P, et al. Heritability analysis of spherical equivalent, axial length, corneal curvature, and anterior chamber depth in the Beaver Dam Eye Study. *Arch Ophthalmol.* 2009;127(5):649–655.
3. Wojciechowski R, Congdon N, Bowie H, Munoz B, Gilbert D, West SK. Heritability of refractive error and familial aggregation of myopia in an elderly American population. *Invest Ophthalmol Vis Sci.* 2005;46(5):1588–1592.
4. Biino G, Palmas MA, Corona C, et al. Ocular refraction: heritability and genome-wide search for eye morphometry traits in an isolated Sardinian population. *Hum Genet.* 2005;116(3):152–159.
5. Lyhne N, Sjolie AK, Kyvik KO, Green A. The importance of genes and environment for ocular refraction and its determiners: a population based study among 20–45 year old twins. *Br J Ophthalmol.* 2001;85(12):1470–1476.
6. Teikari JM, O'Donnell J, Kaprio J, Koskenvuo M. Impact of heredity in myopia. *Hum Hered.* 1991;41(3):151–156.
7. Hammond CJ, Snieder H, Gilbert CE, Spector TD. Genes and environment in refractive error: the twin eye study. *Invest Ophthalmol Vis Sci.* 2001;42(6):1232–1236.
8. Curtin BJ. *The Myopias: Basic Science and Clinical Management.* Philadelphia: Harper & Row; 1985.
9. Rada JA, Shelton S, Norton TT. The sclera and myopia. *Exp Eye Res.* 2006;82(2):185–200.
10. Butler GS, Overall CM. Updated biological roles for matrix metalloproteinases and new "intracellular" substrates revealed by degradomics. *Biochemistry.* 2009;48(46):10830–10845.
11. Malemud CJ. Matrix metalloproteinases (MMPs) in health and disease: an overview. *Front Biosci.* 2006;11:1696–1701.
12. Chakraborti S, Mandal M, Das S, Mandal A, Chakraborti T. Regulation of matrix metalloproteinases: an overview. *Mol Cell Biochem.* 2003;253(1–2):269–285.
13. Hall NF, Gale CR, Ye S, Martyn CN. Myopia and polymorphisms in genes for matrix metalloproteinases. *Invest Ophthalmol Vis Sci.* 2009;50(6):2632–2636.
14. Guggenheim JA, McBrien NA. Form-deprivation myopia induces activation of scleral matrix metalloproteinase-2 in tree shrew. *Invest Ophthalmol Vis Sci.* 1996;37(7):1380–1395.
15. Liu SZ, Wu WC, Wang JF, Tan XP, Jiang HB. Dynamic changes of MMP-2 activity in the posterior sclera of chicks with form-deprivation myopia (in Chinese). *Zhong Nan Da Xue Xue Bao Yi Xue Ban.* 2005;30(3):295–298.
16. Siegwart JT Jr, Norton TT. The time course of changes in mRNA levels in tree shrew sclera during induced myopia and recovery. *Invest Ophthalmol Vis Sci.* 2002;43(7):2067–2075.
17. Siegwart JT Jr, Norton TT. Selective regulation of MMP and TIMP mRNA levels in tree shrew sclera during minus lens compensation and recovery. *Invest Ophthalmol Vis Sci.* 2005;46(10):3484–3492.
18. Stambolian D, Ciner EB, Reider LC, et al. Genome-wide scan for myopia in the Old Order Amish. *Am J Ophthalmol.* 2005;140(3):469–476.
19. Stambolian D, Ibay G, Reider L, et al. Genomewide linkage scan for myopia susceptibility loci among Ashkenazi Jewish families shows evidence of linkage on chromosome 22q12. *Am J Hum Genet.* 2004;75(3):448–459.
20. Wojciechowski R, Stambolian D, Ciner E, Ibay G, Holmes TN, Bailey-Wilson JE. Genomewide linkage scans for ocular refraction and meta-analysis of four populations in the Myopia Family Study. *Invest Ophthalmol Vis Sci.* 2009;50(5):2024–2032.
21. Kong X, Matise TC. MAP-O-MAT: internet-based linkage mapping. *Bioinformatics.* 2005;21(4):557–559.
22. O'Connell JR, Weeks DE. PedCheck: a program for identification of genotype incompatibilities in linkage analysis. *Am J Hum Genet.* 1998;63(1):259–266.
23. Wigginton JE, Abecasis GR. PEDSTATS: descriptive statistics, graphics and quality assessment for gene mapping data. *Bioinformatics.* 2005;21(16):3445–3447.
24. Broman KW, Weber JL. Estimation of pairwise relationships in the presence of genotyping errors. *Am J Hum Genet.* 1998;63(5):1563–1564.
25. Abecasis GR, Cherny SS, Cookson WO, Cardon LR. Merlin: rapid analysis of dense genetic maps using sparse gene flow trees. *Nat Genet.* 2002;30(1):97–101.
26. Chen WM, Abecasis GR. Family-based association tests for genome-wide association scans. *Am J Hum Genet.* 2007;81(5):913–926.
27. Gabriel SB, Schaffner SF, Nguyen H, et al. The structure of haplotype blocks in the human genome. *Science.* 2002;296(5576):2225–2229.
28. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics.* 2005;21(2):263–265.
29. Kall L, Storey JD, Noble WS. QVALITY: non-parametric estimation of q-values and posterior error probabilities. *Bioinformatics*. 2009;25(7):964–966.
30. Storey JD. A direct approach to false discovery rates. *J R Stat Soc Ser B.* 2002;64(3):19.
31. Ben-Simon GJ, Peiss M, Anis E, Nakra T, Luski A, Spierer A. Spectacle use and reduced unaided vision in third grade students: a comparative study in different educational settings. *Clin Exp Optom.* 2004;87(3):175–179.
32. Zylbermann R, Landau D, Berson D. The influence of study habits on myopia in Jewish teenagers. *J Pediatr Ophthalmol Strabismus.* 1993;30(5):319–322.
33. Seko Y, Azuma N, Takahashi Y, et al. Human sclera maintains common characteristics with cartilage throughout evolution. *PLoS One.* 2008;3(11):e3709.
34. Siegwart JT Jr, Norton TT. Steady state mRNA levels in tree shrew sclera with form-deprivation myopia and during recovery. *Invest Ophthalmol Vis Sci.* 2001;42(6):1153–1159.
35. Rada JA, Brenza HL. Increased latent gelatinase activity in the sclera of visually deprived chicks. *Invest Ophthalmol Vis Sci.* 1995;36(8):1555–1565.
36. Liang CL, Wang HS, Hung KS, et al. Evaluation of MMP3 and TIMP1 as candidate genes for high myopia in young Taiwanese men. *Am J Ophthalmol.* 2006;142(3):518–520.
37. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev.* 2000;14(2):163–176.
38. Jenkins G. The role of proteases in transforming growth factor-beta activation. *Int J Biochem Cell Biol.* 2008;40(6–7):1068–1078.
39. Lin HJ, Wan L, Tsai Y, et al. The TGFbeta1 gene codon 10 polymorphism contributes to the genetic predisposition to high myopia. *Mol Vis.* 2006;12:698–703.
40. Lin HJ, Wan L, Tsai Y, et al. Sclera-related gene polymorphisms in high myopia. *Mol Vis.* 2009;15:1655–1663.
41. Janssen A, Hoellenriegel J, Fogarasi M, et al. Abnormal vessel formation in the choroid of mice lacking tissue inhibitor of metalloprotease-3. *Invest Ophthalmol Vis Sci.* 2008;49(7):2812–2822.
42. Gaton DD, Sagara T, Lindsey JD, Weinreb RN. Matrix metalloproteinase-1 localization in the normal human uveoscleral outflow pathway. *Invest Ophthalmol Vis Sci.* 1999;40(2):363–369.
43. Young TL, Guo XD, King RA, Johnson JM, Rada JA. Identification of genes expressed in a human scleral cDNA library. *Mol Vis.* 2003;9:508–514.
44. Young TL, Scavello GS, Paluru PC, Choi JD, Rappaport EF, Rada JA. Microarray analysis of gene expression in human donor sclera. *Mol Vis.* 2004;10:163–176.
45. Mignatti P, Rifkin DB. Plasminogen activators and matrix metalloproteinases in angiogenesis. *Enzyme Protein.* 1996;49(1–3):117–137.
46. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. *Endocr Rev.* 2002;23(6):824–854.
47. Zhu X, Wallman J. Opposite effects of glucagon and insulin on compensation for spectacle lenses in chicks. *Invest Ophthalmol Vis Sci.* 2009;50(1):24–36.
48. Rajah R, Katz L, Nunn S, Solberg P, Beers T, Cohen P. Insulin-like growth factor binding protein (IGFBP) proteases: functional regulators of cell growth. *Prog Growth Factor Res.* 1995;6(2–4):273–284.