

Lack of association with high myopia and the MYP2 locus in the Japanese population by high resolution microsatellite analysis on chromosome 18

Takahiro Yamane¹
 Jeewon Mok²
 Akira Oka²
 Eiichi Okada³
 Ritsuko Nishizaki^{1,3}
 Akira Meguro¹
 Junichi Yonemoto¹
 Jerzy K Kulski^{2,4}
 Shigeaki Ohno⁵
 Hidetoshi Inoko²
 Nobuhisa Mizuki¹

¹Department of Ophthalmology, Yokohama City University School of Medicine, Yokohama, Kanagawa, Japan; ²Department of Basic Medical Science and Molecular Medicine, Tokai University School of Medicine, Isehara, Kanagawa, Japan; ³Okada Eye Clinic, Yokohama, Kanagawa, Japan; ⁴Centre for Comparative Genomics, School for Information Technology, Murdoch University, Murdoch, Western Australia, Australia; ⁵Department of Ophthalmology, Hokkaido University School of Medicine, Sapporo, Hokkaido, Japan

Correspondence: Nobuhisa Mizuki
 Department of Ophthalmology, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawa, Yokohama 236-0004, Japan
 Tel +81 457872683
 Fax +81 457819755
 Email mizunobu@med.yokohama.cu.jp

Abstract: MYP2 was reported for a candidate locus associated with high grade myopia by linkage analysis, but no candidate gene has been detected. We report an association study in the Japanese population using 750 microsatellite markers on chromosome 18 that include MYP2 locus. 450 Japanese subjects with high myopia whose refractive error was greater than or equal to -9.25D in at least one eye and equal number of normal control subjects were recruited in this study. Three steps screening on the pooled DNA of patients and the pooled DNA of controls were performed in this study. A total of 722 microsatellite markers could be analyzed, and we obtained 4 positive markers. Then to avoid experimental errors and artifacts, we confirmed true allele frequency by individual genotyping using initial set of 450 patients and controls. Only marker D18S0301i showed statistically significance, and no marker showed statistically significance on the MYP2 locus. Near the marker D18S0301i, GALNT1 gene was located, but its relation to high myopia has remained to be identified.

Keywords: myopia, MYP2, microsatellite mapping, chromosome18

Introduction

Myopia is a refractive visual disorder often associated with retinal detachment, submacular hemorrhage, glaucoma, and macular degeneration (Burton 1989). Therefore myopia is an important public health problem because it increases the risk of visual loss and places a social, economic and health burden on the community. In Asian countries including Japan, the prevalence of myopia is very high. For instance, in Japan ~59.3% of the high school students were affected by myopia (Ministry of Education, Japan 2004). High grade myopia occupies the high rank of the causes of midway blindness for people during their lifespan.

The cause of myopia is unclear, but the importance of environmental factors in the prevalence and the progress of the myopia has been well demonstrated by the experiment myopia animal models (Wiesel and Raviola 1977; Wallman et al 1978; Hodos et al 1985; Raviola and Wiesel 1985; Osol et al 1986; Schaeffel et al 1988; Hung et al 1995). The influence of the genetic factors in the development of high myopia has also been demonstrated in epidemiological studies, such as the family correlated studies and twin studies. Although heritability as a measure of estimating the extent to which the genetic factor affects the etiology of a particular disease can be easily influenced by the environment, the heritability of myopia in the twin studies has shown considerably high values (Hu 1981; Teikari et al 1991; Angi et al 1993; Hammond et al 2001; Lyhne et al 2001).

Another measurement that can estimate the extent to which a genetic factor affects the etiology of disease is the concept of λ_R (Risch 1990). Whereas λ_R is defined as the risk ratio for a type R relative of an affected individual compared with the population prevalence, λ_s is a value that divides the proband's sib's prevalence of disease with

the prevalence of disease of the general population. In this regard, such analyses have confirmed that genetics plays an important role in the pathogenesis of high myopia. For example, Guggenheim et al (2000), in a Danish epidemiological investigation (Goldschmidt 1968), calculated that the λ_s was 20.0 for high myopia, where the refractive error was greater than or equal to -6 diopters(D).

Recent multigenerational linkage studies have reported several candidate loci of high myopia, MYP2-MYP5, and MYP11-MYP13 (Young, Ronan, Alvear et al 1998; Young, Ronan, Drahozal et al 1998; Naiglin et al 2002; Paluru et al 2003, 2005; Zhang et al 2005, 2006), and several candidate loci of myopia, MYP6-MYP10 (Stambolian et al 2004; Hammond et al 2004) on different chromosomes. Among them, we focused on the MYP2 locus on chromosome 18 that showed the highest maximum lod score (9.59). Previous studies reported that the MYP2 locus was the candidate locus of the nonsyndromic autosomal dominant high myopia although these mapping resolution were insufficient.

In this study, to clarify the susceptibility genes on chromosome 18 related to the appearance of high myopia, we performed high resolution mapping by association study using 750 polymorphic microsatellite markers distributed across chromosome 18.

Materials and methods

A total of 450 Japanese patients with high myopia whose refractive error was greater than or equal to -9.25 D in at least one eye were recruited in the Hospital of Yokohama City University and Okada Eye Clinic. Equal numbers of control subjects were recruited at the Tokai University, Isehara campus. All the patients with high myopia were examined clinically by experienced ophthalmologists, and none of the patients were found to have eye diseases, such as Marfan syndrome, Stickler syndrome, juvenile glaucoma and keratoconus. Control subjects were selected from the general population. The average age was 37.8 ± 11.77 years in the patient population and the gender ratio was 1.0:1.4 (male: female). In the control population, average age was 41.2 ± 11.67 years and gender ratio was 1.0:1.2 (male: female). The average spherical refractive error for patients was OD: -11.63 ± 2.21 D (range -6.75 to -22.75 D), OS: -11.58 ± 2.21 D (range -7.25 to -22.50 D). The average axial length was OD: 27.78 ± 1.31 mm (range 20.31 to 33.14 mm), OS: 27.79 ± 1.33 mm (range 23.99 to 34.74 mm). The average corneal refraction was OD: 43.94 ± 1.60 D (range 39.50 to 50.25D), OS: 43.94 ± 1.62 D (range 39.75 to 53.00D).

Peripheral blood cells were collected and consent to genetic screening was obtained from patients and control

subjects after the details of this study were explained to every subject. All patients and healthy control subjects agreed to a blood examination conducted according to the guidelines of the Declaration of Helsinki.

Based on the knowledge that the average length of linkage disequilibrium (LD) is about 100 kb (Tamiya et al 2005), we performed high resolution screening on chromosome 18, including the MYP2 locus, using 750 microsatellite markers. The average interval length between the markers was 126.8 kb. Information on the microsatellites markers was obtained from the JBIRC website (Japan Biological Information Research Center).

In order to reduce the cost of genotyping and the total number of genotyping assays and associated costs, we performed three screens on the pooled DNA of patients and the pooled DNA of the controls, separately (Sham et al 2002). The three-step screening protocol, as previously described (Tamiya et al 2005), helped to decrease the number of false positives. We used 150 myopia individuals and 150 controls at each step of the screening protocol to construct the pooled DNA samples. The frequency of the alleles in the pooled DNA were calculated by obtaining the sum total of the height of all peak patterns after an electrophoresis run by ABI 3700 DNA analyzer (Applied Biosystems) and calculating the ratio of the height of each peak.

In the first screening step, 750 microsatellite markers were analyzed using pooled DNA. In the second screening step, only the positive markers from the first screening with statistical significance of $p < 0.05$ by the Fisher's exact test, either for the 2×2 or $2 \times m$ contingency table (m refers to the number of allele), were genotyped using the pooled DNA that was constructed from another 150 myopia individuals and 150 controls. Only positive markers showing significance with $p < 0.05$ in the second screening step were then subjected to the third screening step. In this way, the three step screening method could eliminate false positives resulting from type 1 errors.

Finally, in order to further exclude experimental errors and artifacts, we selected priority microsatellite markers based on statistical p -value and peak pattern to confirm true allele frequency by individual genotyping using the DNA samples of the initial set of 450 high myopia individuals and 450 control individuals.

DNA was extracted by using the QIAamp DNA blood kit (QIAGEN) and the DNA concentration was measured using the PicoGreen fluorescence assay. Because accurate DNA concentration was very important in the use of the pooled DNA method, measurements were performed in triplicate.

All microsatellite marker specific PCR primers were designed to have an annealing temperature of 57°C , and forward primers were labeled with fluorogenic probe.

The volume of the PCR mixture for pooled DNA typing was a total of 20 µl containing 24 ng of pooled DNA, 0.5 units of AmpliTaq-Gold DNA polymerase (Applied Biosystems), 5.0 mM dNTPs, 20 µM of each primers and 1X reaction buffer. The PCR mixture for individual typing was a total of 20 µl containing 8 ng of genomic DNA, 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems), 4.0 mM dNTPs, 1X reaction buffer, 20 µM of each primers and 1X reaction buffer. The amplification reactions were carried out in GeneAmp PCR system 9700 (Applied Biosystems) with an initial denaturation at 96 °C for 5 min (hot start), annealing at 57 °C for 1 min, and extension at 72 °C for 1 min and then 40 cycles for three steps of PCR (denaturation at 96 °C for 45 s, and annealing at 57 °C for 45 s, and extension at 72 °C for 1 min).

The quality of the pooled DNA was confirmed by comparing the result of allelic distributions between pooled DNA typing and individual typing using 6 microsatellite markers that had no association with high myopia. Both pooled DNA typing and individual typing were carried out using the ABI 3700 DNA analyzer (Applied Biosystems).

Results

Of the 722 microsatellite markers analyzed in the first screening step, 94 markers were calculated to be positive markers (Figure 1). Four of the 94 markers were excluded for the second screening step because they could not be amplified, or had a very weak peak pattern.

Therefore, in the second screening step, 90 positive markers from the first screening were analyzed and only 16 of them were found to be positive for subsequent use in a third screening. In the third screening, we obtained only 4 positive markers (D18S0356i, D18S0201i, D18S0301i, D18S0357i) with statistical significance of $p < 0.05$ by Fisher's exact test (Table 1).

To exclude experimental errors, we then checked again all the peak patterns of the 4 positive markers, and finally selected the three markers, D18S0356i, D18S0201i and D18S0301i, for further analysis by individual genotyping in patients and controls. The marker, D18S0357i, was excluded from the list for individual typing, because the results were not consistent for each step of the screening protocol and a variability of certain, peak patterns between the three steps.

Table 2 shows the result of individual typing for the three selected markers. Only marker D18S0301i that was located at 18q12.2, showed statistically significance ($p = 0.038$ 2×2 , OR = 0.61, 95% CI = 0.383 to 0.973) at the allele which

Table 1 Three step screening of pooled DNA method

Registration ID	5' primer sequence 3' primer sequence	p-values					
		1st		2nd		3rd	
		2×2	$2 \times m$	2×2	$2 \times m$	2×2	$2 \times m$
D18S0356i	TCTTCAATAAGCAACTGAATCT CCTAAATGAATTCATAACCTAGTTT	0.0204802	0.0186256	0.0200699	0.0407851	0.00827632	0.00552037
D18S0201i	CAGAGCTTAGTCATGTCAGAC ATTAGTTATCTGAAGTTGCTT	0.00164119	0.0392785	0.0000000017854	0.000000213675	0.0000742732	0.00767778
D18S0301i	GAGTAAAGCTAGGATCCAGAG AGTAACTTCTGATCCATCCT	0.0155871	0.0155871	0.000038454	0.000038454	0.0256271	0.0256271
D18S0357i	GAGCTTACAGTCAGCTGAGAT CAGGTGACAAATTATATAGCTGAT	0.000000568913	0.000000953615	0.0318655	0.0418926	0.0255968	0.0604102

p values were calculated by Fisher's exact test, based on 2×2 and $2 \times m$ contingency tables with estimated allele frequencies. The smallest P value was selected

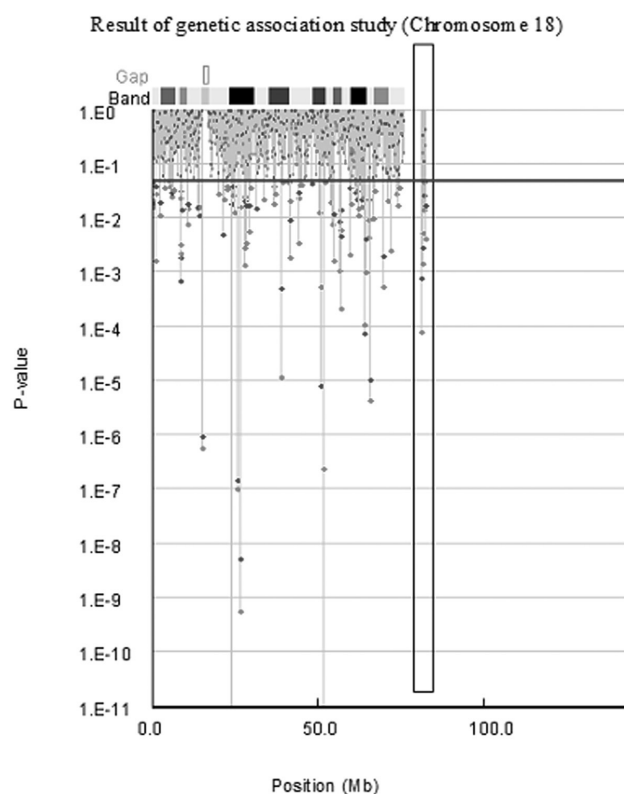


Figure 1 Shows p values by Fisher's exact test based on 2×2 contingency tables (Red circles) or on $2 \times m$ contingency tables (Blue circles) in the first screening. Blue line indicate p values = 0.05.

has amplicon size 461. The marker D18S0201i, that was located in the MYP2 locus between D18S59 and D18S1146, showed no statistically significance.

Discussion

MYP2 is a candidate locus of the nonsyndromic autosomal dominant high myopia first identified by Young, Ronan, Drahozal et al (1998) who performed a genome-wide linkage analysis for myopia susceptibility loci in 8 multigenerational families with an autosomal dominant mode of myopia of more than -6.00 diopters, and showed a significant linkage to 18p. The maximum lod score was 9.59, with marker D18S481. Haplotype analysis further refined this myopia locus to a 7.6-cM interval between markers D18S59 and D18S1138 on 18p11.31. Afterwards Young et al (2001) narrowed the candidate region to the interval of 0.8 cM between markers D18S63 and D18S52. In this region, there are many possible candidate genes, such as adenylate cyclase-activating polypeptide, thymidylate synthetase, protein tyrosine phosphatase receptor, α subunit of guanine nucleotide-binding protein, protein tyrosine phosphatase, Niemann-Pick disease protein, and the α subunit of laminin.

The transforming growth factor- β -induced factor (TGIF) which was mapped to the MYP2 region on 18p11.3, also

Table 2 Statistical significance of alleles associated with high myopia on chromosome 18

Registration ID	No. of alleles	Allele amplicon size	Allele frequencies		p-values	OR	95%CI
			Patient	Control			
D18S0356i	7	431	0.040	0.032	0.4486		
		435	0.006	0.002	0.4522		
		439	0.324	0.333	0.7246		
		443	0.376	0.412	0.1335		
		447	0.219	0.188	0.1127		
		451	0.034	0.031	0.8939		
		455	0.001	0.001	1		
D18S0201i	10	401	0.017	0.017	1		
		427	0.000	0.002	0.4997		
		429	0.010	0.003	0.0894		
		431	0.351	0.379	0.231		
		433	0.069	0.051	0.1274		
		435	0.271	0.272	0.9569		
		437	0.207	0.186	0.3036		
		439	0.045	0.043	0.815		
		441	0.028	0.041	0.1482		
		443	0.001	0.005	0.3743		
D18S0301i	4	458	0.000	0.001	0.4994		
		461	0.033	0.054	0.0376	0.61	0.383–0.973
		464	0.962	0.943	0.0596		
		467	0.004	0.002	0.687		

p values were calculated by Fisher's exact test, based on 2×2 contingency tables.

emerged as a candidate gene for high myopia. Subsequently, Lam et al (2003) investigated the coding exons of TGIF for mutations in Chinese subjects with high myopia, and only 657 (T-G) showed statistical significance in the logistic regression model (odds ratio 0.133; 95% CI 0.036–0.488; $p = 0.002$). Although TGIF was suggested as a probable candidate gene for high myopia by Lam et al (2003), others showed that TGIF has no association with high myopia (Scavell 2004; Hasumi 2006). In our present high resolution screening study, we used a total set of 35 microsatellite markers in the MYP2 locus between markers D18S59 to D18S52, and found no association between MYP2 and high myopia. Therefore, based on our study, we conclude that there is no candidate gene for high myopia in the MYP2 region in the Japanese population.

On the other hand, we detected another candidate locus for high myopia outside the MYP2 region on chromosome 18. The microsatellite marker D18S0301i located in chromosomal position q12.2, showed statistical significance (odds ratio 0.61; 95%CI 0.383–0.973; $p = 0.038$). To avoid false negative, we performed no correction for multiple comparisons such as Bonferroni's correction in this study.

The gene closest to this microsatellite marker is, GALNT1 (UDP-N-acetyl- α -D-galactosamine). UDP-N-acetyl- α -D-galactosamine is a N-acetylgalactosaminyltransferase that transfers an N-acetylgalactosamine to a serine or threonine residue in the first step of O-linked oligosaccharide biosynthesis (Takai et al 1997). We considered GALNT1 as a new candidate gene, but its relation to myopia is debatable because of its metabolic function. There is still a possibility that a specific allele or mutation in an unknown gene and near to the GALNT1 gene is in linkage disequilibrium with marker D18S0301i and has yet to be identified. Therefore, we may need to investigate this locus in more detail in future studies.

Since the accumulation of several multigenic factors may contribute to the risk and development of chronic diseases, we plan to expand our investigations to other chromosomes of the human genome, in order to identify all the disease susceptibility alleles or mutations involved with high myopia at the genome-wide level.

References

- Angi MR, Clementi M, Sardei C, et al. 1993. Heritability of myopic refractive errors in identical and fraternal twins. *Graefes Arch Clin Exp Ophthalmol*, 231:580–5.
- Burton TC. 1989. The influence of refractive error and lattice degeneration on the incidence of RD. *Trans Am Ophthalmol Soc*, 87:143–55.
- Goldschmidt E. 1968. On The etiology of myopia. *Acta Ophthalmol*, Suppl 98.
- Guggenheim JA. 2000. The heritability of high myopia: a reanalysis of Goldschmidt's data. *J Med Genet*, 37:227–31.
- Hammond CJ, Snieder H, Gilbert CE, et al. 2001. Genes and environment in refractive error: the twin eye study. *Invest Ophthalmol Vis Sci*, 42:1232–6.
- Hammond CJ, Andrew T, Mak YT, et al. 2004. A susceptibility locus for myopia in the normal population is linked to the PAX6 gene region on chromosome 11: a genomewide scan of dizygotic twins. *Am J Hum Genet*, 75:294–304.
- Hasumi Y, Inoko H, Mano S, et al. 2006. Analysis of single nucleotide polymorphisms at 13 loci within the transforming growth factor-induced factor gene shows no association with high myopia in Japanese subjects. *Immunogenetics*, 58:947–53.
- Hodos W, Fitzke FW, Hayes BP, et al. 1985. Experimental myopia in chicks: ocular refraction by electroretinography. *Invest Ophthalmol Vis Sci*, 26:1423–30.
- Hu DN. 1981. Twin study on myopia. *Chin Med J (Engl)*, 94:51–5.
- Hung LF, Crawford ML, Smith EL. 1995. Spectacle lenses alter eye growth and refractive status of young monkeys. *Nat Med*, 1:761–5.
- Lam DS, Lee WS, Leung YF, et al. 2003. TGF β -induced factor: a candidate gene for high myopia. *Invest Ophthalmol Vis Sci*, 44:1012–5.
- Lyhne N, Sjolie AK, Kyvik KO, et al. 2001. 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*, 85:1470–6.
- Ministry of Education, Culture, Sports, Science and Technology. Disease Rate among Students, Statistical Abstract. 2004 edition.
- Naiglin L, Gazagne C, Dallongeville F, et al. 2002. A genome wide scan for familial high myopia suggests a novel locus on chromosome 7q36. *J Med Genet*, 39:118–24.
- Osol G, Schwarz B, Foss DC. 1986. The effects of photoperiod and lid suture on eye growth in chickens. *Invest Ophthalmol Vis Sci*, 27:255–60.
- Paluru P, Ronan SM, Heon E, et al. 2003. New locus for autosomal dominant high myopia maps to the long arm of chromosome 17. *Invest Ophthalmol Vis Sci*, 44:1830–6.
- Paluru PC, Nallasamy S, Devoto M, et al. 2005. Identification of a novel locus on 2q for autosomal dominant high-grade myopia. *Invest Ophthalmol Vis Sci*, 46:2300–7.
- Raviola E, Wiesel TN. 1985. An animal model of myopia. *N Engl J Med*, 312:1609–15.
- Risch N. 1990. Linkage strategies for genetically complex traits. I. Multi-locus models. *Am J Hum Genet*, 46:222–8.
- Scavell GS, Paluru PC, Ganter WR, et al. 2004. Sequence variants in the transforming growth factor-induced factor (TGIF) gene are not associated with high myopia. *Invest Ophthalmol Vis Sci*, 45:2091–7.
- Schaeffel F, Glasser A, Howland HC. 1988. Accommodation, refractive error and eye growth in chickens. *Vision Res*, 28:639–57.
- Sham P, Bader JS, Craig I, et al. 2002. DNA Pooling: a tool for large-scale association studies. *Nat Rev Genet*, 3:862–71.
- Stambolian D, Ibay G, Reider L, et al. 2004. Genomewide linkage scan for myopia susceptibility loci among Ashkenazi Jewish families shows evidence of linkage on chromosome 22q12. *Am J Hum Genet*, 75:448–59.
- Takai S, Hinoda Y, Adachi T, et al. 1997. A human UPD (sic)-GalNAc: polypeptide, N-acetylgalactosaminyltransferase type 1 gene is located at the chromosomal region 18q12.1. *Hum Genet*, 99:293–4.
- Tamiya G, Shinya M, Imanishi T, et al. 2005. Whole genome association study of rheumatoid arthritis using 27039 microsatellites. *Hum Mol Genet*, 14:2305–21.
- Teikari JM, O'Donnell J, Kaprio J, et al. 1991. Impact of heredity in myopia. *Hum Hered*, 41:151–6.
- Wallman J, Turkel J, Trachtman J. 1978. Extreme myopia produced by modest change in early visual experience. *Science*, 201:1249–51.
- Wiesel TN, Raviola E. 1977. Myopia and eye enlargement after neonatal lid fusion in monkeys. *Nature*, 266:66–8.
- Young TL, Atwood LD, Ronan SM, et al. 2001. Further refinement of the MYP2 locus for autosomal dominant high myopia by linkage disequilibrium analysis. *Ophthalmic Genet*, 22:69–75.
- Young TL, Ronan SM, Alvear AB, et al. 1998. A second locus for familial myopia maps to chromosome 12q. *Am J Hum Genet*, 63:1419–24.

- Young TL, Ronan SM, Drahozal LA, et al. 1998. Evidence that a locus for familial high myopia maps to chromosome 18p. *Am J Hum Genet*, 63:109–19.
- Zhang Q, Guo X, Xiao X, et al. 2005. A new locus for autosomal dominant high myopia maps to 4q22–q27 between D4S1578 and D4S1612. *Molec Vis*, 11:554–60.
- Zhang Q, Guo X, Xiao X, et al. 2006. Novel locus for X linked recessive high myopia maps to Xq23–q25 but outside MYP1. *J Med Genet*, 43:e20. Note: Electronic Article.