A single nucleotide polymorphism analysis of the \textit{LAMA1} gene in Japanese patients with high myopia

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\textbf{Abstract:} Although a myopia susceptibility gene has not yet been elucidated, ten candidate regions (MYP1–MYP10) have been associated with myopia by linkage analysis employing large pedigrees. We report herein on the results of our analysis pertaining to polymorphisms of \textit{LAMA1} (alpha subunit of laminin), a promising candidate gene for high myopia present in the MYP2 region of Japanese subjects with high myopia. Three hundred and thirty Japanese subjects with high myopia at a level of greater than \(-9.25\) D and ethnically and sex matched 330 normal controls without high myopia was enrolled in this study. The thirteen SNPs located on the \textit{LAMA1} gene were analyzed using PCR and SNP-specific fluorogenic probes. Two of the SNPs were monomorphic and none of the 11 SNPs showed statistically significant association with high myopia in the Japanese population. There is no convincing evidence to prove a connection between nucleotide sequence variations in \textit{LAMA1} and high myopia. The pairwise linkage disequilibrium (LD) mapping disclosed a strong value (\(D' > 0.8\)) and narrow ranged block within these SNPs.

\textbf{Introduction}

Myopia affects about 25% of the adult population in the United States (Curtin 1970, 1985; Leibowitz et al 1980; Burton 1990; Katz et al 1997) and is considered a significant public health problem, especially in Asian populations such as Chinese and Indians. Japan has one of the highest rates of myopia in the world, affecting \(-59.3\%\) of the high school students (MEXT 2004). High myopia can cause blindness or a severe loss of visual acuity due to retinal detachment, submacular hemorrhage, glaucoma, or macular degeneration (Burton 1990). However, effective treatment methodology and preventive strategies for high myopia have not yet been fully established.

Experimental and epidemiological studies have both indicated the importance of environmental and genetic factors in the development of myopia. In recent years, a wide array of experimental studies on animals, such as young monkeys and chicks, have shown that myopization occurs following ocular axial elongation induced by surgical eyelid closure (form deprivation experimental myopia) or imposing with a strong concave lens (lens induced experimental myopia) (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) pointing to the importance of the environmental factor in the development of myopia. As well, It has also been indicated from epidemiological studies that the prevalence rate of myopia varies with environmental factors, such as the time spent on near work (Saw et al 2002), level of education (Rosner and Belkin 1987; Wedner et al 2002; Wong et al 2002; Shimizu et al 2003) and lifestyle (Gardner et al 1999; Zhan et al 2000; Dandona et al 2002). However, von Noorden et al (1978) reported that in monkeys with their eyelids sutured in the same fashion,
there were those that became myopic as well as those that did not differ in their responses or in the pathogenesis of myopia. This study inferred the contributions of both genetic factors and environmental factors in the growth process of the eye after birth.

The significance of genetic factors has long been indicated in the development and progression of myopia, and in particular, it is held that the more severe the degree of myopia is, the stronger the impact of the genetic factor becomes. As a measure estimating the extent to which the genetic type is affecting the pathogenesis of a particular disease, there is the concept of heritability. For example, several twin studies have been conducted in myopia showing high heritability that has inferred a strong contribution by genetic factors to myopia (Hu 1981; Teikari et al 1991; Angi et al 1993; Hammond et al 2001; Lyhne et al 2001). Recently, family linkage studies on autosomal dominant high myopia have associated at least ten myopia susceptibility genomic loci (MYP1–MYP10) candidates with the occurrence of myopia (Young, Ronan, Alvear et al 1998; Young, Ronan, Drahozal et al 1998; Schwartz et al 1990; Naiglin L et al 2002; Paluru et al 2003). Among them, MYP1–5 is thought to be strongly involved in the pathogenesis of high myopia. MYP2 was the candidate region for an autosomal dominant high myopia gene, showing the maximum lod score of 9.59 (Young et al 1998). The MYP2 locus spreads across the 7.6 cM genomic region on the short arm of the chromosome 18. This candidate region contained nine potentially relevant genes clusterin-like 1 (CLUL1), elastin microfibrillar interfacer 2 (EMILIN2), lipin region contained nine potentially relevant genes clusterin-like region on the short arm of the chromosome 18. This candidate region for an autosomal dominant high myopia gene, showing the maximum lod score of 9.59 (Young, Ronan, Alvear et al 1998; Young, Ronan, Drahozal et al 1998; Schwartz et al 1990; Naiglin L et al 2002; Paluru et al 2003). Among them, MYP1–5 is thought to be strongly involved in the pathogenesis of high myopia. MYP2 was the candidate region for an autosomal dominant high myopia gene, showing the maximum lod score of 9.59 (Young et al 1998). The MYP2 locus spreads across the 7.6 cM genomic region on the short arm of the chromosome 18. This candidate region contained nine potentially relevant genes clusterin-like 1 (CLUL1), elastin microfibrillar interfacer 2 (EMILIN2), lipin region contained nine potentially relevant genes clusterin-like region on the short arm of the chromosome 18. This candidate region contained nine potentially relevant genes clusterin-like 1 (CLUL1), elastin microfibrillar interfacer 2 (EMILIN2), lipin region contained nine potentially relevant genes clusterin-like region on the short arm of the chromosome 18. This candidate region contained nine potentially relevant genes clusterin-like 1 (CLUL1), elastin microfibrillar interfacer 2 (EMILIN2), lipin 2 (LPIN2), myomesin 1 (MYOM1), myosin regulatory light chain 3 (MIRC3), myosin regulatory light chain 2 (MRLC2), transforming growth β-induced factor (TGIFβ), large Drosophila homolog association protein 1 (DLGPA1), and zinc finger protein 161 homolog (ZFP161). Scavello et al (2005) checked mutations of these 9 encoded positional candidate genes by direct sequencing; although no particular sequence alternations were identified in disease phenotype. Any other implicated genes as the causative need to be surveyed adjacent to MYP2.

Laminin is a major component of basement membranes and is a glycoprotein of 900 kD with multiple domain structures. Its three component chains, α, β and γ are bonded to each other with the α chain being in the central region and they form a cruciform structure (Paulsson et al 1985). Laminin is present in the basement membrane on the side adjacent to cells and is thought to be involved in cell adhesion, differentiation and migration. Laminin is present throughout the sclera in association with basement membranes of blood vessels, the trabecular meshwork, and Schlemm’s canal (Dietlein et al 1998). Additionally, laminin has been located to the surface of cribiform plates of the lamina cribrosa of human sclera (Hernandes et al 1987). As such, laminin is an integral constituent of the sclera and is a major component of the corneal epithelial basement membrane as well. The gene encoding the α chain of laminin is LAMA1 gene which is reported to be located at the short arm of chromosome 18 (Nagayoshi et al 1989). LAMA1 is composed of 62 exons and its genome size is 175,928 bp. The mRNA size of LAMA1 is 9,530 bp encoding 3075-amino acids (Haaparanta et al 1991; Nissinen et al 1991). The genes encoding the β and γ chains separately reside in chromosomes 7 and 1, respectively. Because the LAMA1 gene lies approximately 1648 kb centromeric of the ZFP161 gene in the MYP2 region, it is considered to be a candidate for the pathogenesis of high myopia.

The association between the SNPs of LAMA1 and high myopia has not been previously reported. Therefore, in this study we, for the first time, studied and sequenced 13 SNPs of the LAMA1 gene among the 330 Japanese patients with high myopia at the level of greater than −9.25 D.

Materials and methods

A total of 330 Japanese subjects with high myopia and ethnically matched unrelated control subjects (n = 330) attending the Ophthalmology Clinic at the Yokohama City University or the Okada eye clinic were recruited for this study. The myopia phenotypes were classified according to the mean spherical equivalent refractive error above the threshold level of −9.25 D sph. The mean refractive error of myopia cases was −11.55 ± 2.17 D sph with a mean axial length of 27.78 ± 1.30 mm. Axial length was measured with the IOL-Master. The mean astigmatism and keratometry of the cases were 1.21 ± 0.88 D and 43.92 ± 1.61 D, respectively. The mean age of case subjects was 37.82 ± 11.97 years (from 14 to 76 years). The male–female ratio of the cases was 0.66:1.00. A group of 330 subjects of same sex with moderate or no myopia (refractive error weaker than −4.0 D) was used as controls. The mean age of control subjects was 40.25 ± 7.6 years. The male−female ratio of the controls was 0.66:1.00. A group of 330 subjects of same sex with moderate or no myopia (refractive error weaker than −4.0 D) was used as controls. The mean age of control subjects was 40.25 ± 7.6 years. The male−female ratio of the controls was 0.66:1.00. A group of 330 subjects of same sex with moderate or no myopia (refractive error weaker than −4.0 D) was used as controls. The mean age of control subjects was 40.25 ± 7.6 years. The male−female ratio of the controls was 0.66:1.00. A group of 330 subjects of same sex with moderate or no myopia (refractive error weaker than −4.0 D) was used as controls. The mean age of control subjects was 40.25 ± 7.6 years. The male−female ratio of the controls was 0.66:1.00. A group of 330 subjects of same sex with moderate or no myopia (refractive error weaker than −4.0 D) was used as controls. The mean age of control subjects was 40.25 ± 7.6 years. The male−female ratio of the controls was 0.66:1.00. A group of 330 subjects of same sex with moderate or no myopia (refractive error weaker than −4.0 D) was used as controls. The mean age of control subjects was 40.25 ± 7.6 years. The male−female ratio of the controls was 0.66:1.00. A group of 330 subjects of same sex with moderate or no myopia (refractive error weaker than −4.0 D) was used as controls. The mean age of control subjects was 40.25 ± 7.6 years. The male−female ratio of the controls was 0.66:1.00. A group of 330 subjects of same sex with moderate or no myopia (refractive error weaker than −4.0 D) was used as controls. The mean age of control subjects was 40.25 ± 7.6 years. The male−female ratio of the controls was 0.66:1.00. A group of 330 subjects of same sex with moderate or no myopia (refractive error weaker than −4.0 D) was used as controls. The mean age of control subjects was 40.25 ± 7.6 years. The male−female ratio of the controls was 0.66:1.00. A group of 330 subjects of same sex with moderate or no myopia (refractive error weaker than −4.0 D) was used as controls. The mean age of control subjects was 40.25 ± 7.6 years. The male−female ratio of the controls was 0.66:1.00. A group of 330 subjects of same sex with moderate or no myopia (refractive error weaker than −4.0 D) was used as controls. The mean age of control subjects was 40.25 ± 7.6 years. The male−female ratio of the controls was 0.66:1.00.
SNP-specific polymerase chain reaction (PCR) primers and fluorogenic probes were designed using ABI (Applied Biosystems). This technique has been utilized extensively in genotyping other candidate genes with multiple single nucleotide polymorphisms. The fluorogenic probes were labeled with a reporter dye (either FAM or VIC) and were specific for one of the two possible bases in the promoter region. A MGB quencher probe was utilized on the 3’ end by a linker arm. TaqMan Universal PCR Master Mix (Applied Biosystems) was used to prepare the PCR. The 2X mix was optimized for TaqMan reactions and contained AmpliTaq-Gold DNA polymerase, dNTPs with UTP and a passive reference dye. Primers, probes, and genomic DNA were added to final concentrations of 300 nmol/l, 100 nmol/l, and 0.5–2.5 ng/μl respectively. Control samples (without DNA template) were run to ensure there was no amplification of contaminating DNA. A reference control DNA was used to verify the identified polymorphisms. The amplification reactions were carried out in an ABI Prism 7700 HT Sequence Detection System (Applied Biosystems GeneAmp® PCR System 9700) with an initial hold step (95 °C for 10 min) and then 40 cycles for the two steps of PCR (denaturation at 92 °C for 15s and annealing at 60 °C for 1 min). The fluorescence intensity of each sample was measured at each temperature change to monitor the amplification of the base pair LAMA1 promoter region. The targeted nucleotide was determined by the fluorescence ratio of the two SNP-specific fluorogenic probes. The fluorescence signal increased when the probe with the exact sequence matched the single stranded template DNA and was digested by the 5’–3’ exonuclease activity of AmpliTaq-Gold DNA polymerase (Applied Biosystems). On digestion, the probe released the fluorescent reporter dye (either FAM or VIC) from the quencher dye.

We used the NCBI dbSNP database (http://www.ncbi.nlm.nih.gov/SNP) to extract the available information on the SNPs in LAMA1 gene. A total of 13 SNPs used in this study were as follows; #rs16950868 in intron56, #rs683733 in exon33, #rs684634 in exon19, #rs334407 in intron1, #rs12454984 in intron1, #rs334420, #rs334384 and #rs2013077 are located in the vicinity of LAMA1 gene (Figure 1). These SNPs were selected using criteria such as population-frequency validation, multiple submitters and high-profile submitters. Either forward or reverse strand sequences of the SNP sites were selected depending on their GC content, to avoid primer dimerization and hairpin structure, and the absence of any other SNP site. The extension reaction was controlled by a mixture of dideoxynucleotides, such that one single-base extension product and one double-base extension product was created corresponding to a SNP allele. This scheme formed two peaks in the mass spectrometer separated by about 300 Da.

We evaluated the allele frequencies of sequence alterations in patients and controls using the Chi–square tests or the Fisher exact tests. Allelic frequencies of all detected SNPs were also assessed for Hardy–Weinberg equilibrium. Statistical analyses were performed on computer using the SPSS software (version 10.1; SPSS Science, Chicago, IL, USA).

The statistical between–group differences were examined using the respective allele models of dominant and recessive. The model of dominant allele 1 is that allele 1 is present on either of the two homologous chromosomes, ie, in this model the onset of disease occurs even when heterozygous for allele 1; this is implied to also be a recessive model for allele 2. The model of recessive allele 1 is that allele 1 is present on both the two homologous chromosomes, ie, in this model the disease does not occur unless homozygous for allele 1; this is implied to also be a dominant model for allele 2.

The strength of LD between SNPs was quantified by Lewontin’s D’ (Lewontin 1964) and haplotype frequencies were estimated from pairs of unphased genotypes by use of EM algorithms in the R package ‘haplo.stats’ (http://www.r-project.org).

Results
We screened 13 SNPs within the the LAMA1 gene for all the cases and controls (Figure 1). Seven SNPs localized in the

![Figure 1](image-url) 13 SNPs analyzed in the LAMA1 gene. Vertical arrows show the 13 SNPs analyzed in this study with the #rs numbers. Left of the figure is 5’ UTR. Black boxes show the translated regions (exons) of this gene with the exon numbers below them.
exons are nonsynonymous substitutions accompanied with an amino acid change. The other 6 SNPs are synonymous changes with four in introns, one in the 5' UTR and one in the 3' UTR of the \textit{LAMA1} gene (Figure 1 and Table 1). Two of the 13 candidate SNP positions rs683733 and rs543355 were monomorphic. No deviations from Hardy-Weinberg equilibrium were observed, except for two monomorphic SNPs by an exact test for Hardy-Weinberg.

Allelic frequencies of 13 SNPs in patients and controls are listed in Table 1. No statistically significant result (Fisher’s exact test, \( p < 0.05 \)) was observed in allele frequencies between the patients and controls (Table 1). Genotype frequencies of these SNPs are shown in Table 2. No significant difference was observed in the genotype frequencies between the patients and the controls.

In relation to the two types of alleles that are present at each SNP (allele 1 and allele 2), statistical between-group differences were examined using their respective allele models of dominant and recessive. No significant between-group differences were noted for the 13 SNPs in any of the dominant and recessive models for each allele.

Figure 2 shows overall LD patterns of the \textit{LAMA1} locus in the patients and the controls. The strong LDs were observed throughout the region from rs334420 to rs12454984, and from rs12961939 to rs67181. These strong LDs were indicated in the same block for myopia patients and controls, but these LD blocks were independent of each other. Association analysis using haplotypes calculated by EM algorithms showed the frequency of haplotype 04 was increased in the patients as compared with the controls (Table 3) (Haplotype 04; OR = 2.46; \( p = 0.01 \)). However, this increase did not reach statistical significance when evaluated by Bonferroni correction.

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### Table 1: Allele frequencies for the 13 SNPs in the \textit{LAMA1} gene among the Japanese patients with high myopia

| SNP rs   | Allele1/2 | Function  | AA change   | Allele frequency (%) | p        |
|----------|-----------|-----------|-------------|----------------------|----------|
|          | Allele 1  | Allele 2  |             | Case                 | Control  |
|          |           |           |             | Allele frequency (%)| Allele 1 | Allele 2 | Allele frequency (%)|                      | Allele 1 | Allele 2 | p        |
| rs334384 | C/T       |           | 's'upstream | 120(18.5)            | 528(81.5)| 112(17.2) | 540(82.8)          | 0.5279 |
| rs334420 | C/T       |           | 's'upstream | 387(59.7)            | 261(40.3)| 398(61.4) | 250(38.6)          | 0.5348 |
| rs334407 | A/G       | Intron1   |             | 263(40.5)            | 387(59.5)| 257(39.7) | 391(60.3)          | 0.7684 |
| rs12454984 | C/T     | Intron6   |             | 54(8.2)              | 602(91.8)| 64(9.8)   | 590(90.2)          | 0.3259 |
| rs684634 | A/G       | Exon11    | Cys⇒Cys     | 548(83.8)            | 106(16.2)| 547(83.4) | 109(16.6)          | 0.8420 |
| rs12961939 | A/C      | Exon33    | Ser⇒Ala     | 604(92.4)            | 50(7.6)  | 611(93.7) | 41(6.3)            | 0.3355 |
| rs12607841 | A/G      | Exon32    | Ala⇒Val    | 48(7.3)              | 608(92.7)| 45(6.9)   | 609(93.1)          | 0.7585 |
| rs11664063 | C/T      | Exon39    | Ala⇒Thr    | 564(86.2)            | 90(13.8) | 562(85.9) | 92(14.1)           | 0.8731 |
| rs671871  | A/G       | Exon44    | Ile⇒Thr    | 577(88.2)            | 77(11.2) | 584(88.9) | 66(10.2)           | 0.3493 |
| rs543355  | C/T       | Exon55    | Thr⇒Ala    | 0(0.0)               | 645(100) | 0(0.0)    | 658(100)           | -     |
| rs683733  | A/C       | Exon56    | Gly⇒Val    | 0(0.0)               | 652(100) | 0(0.0)    | 652(100)           | -     |
| rs16905868 | A/C      | Intron56  |             | 95(14.6)             | 555(85.4)| 105(16.2) | 545(83.8)          | 0.4421 |
| rs2013077 | A/G       | Intron57  |             | 435(66.7)            | 217(33.3)| 422(64.9) | 228(35.1)          | 0.4948 |

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### Discussion

High myopia is a disorder that appears to be caused by the interplay between genetic and environmental factors. As suggested from the analysis of large pedigrees with severe myopia, there exists a multiplicity of genetic risk factors, ie, disease-susceptibility genes, for high myopia, and it is thought to be a multifactorial genetic disorder caused by these factors overlapping each other. For the analysis of the susceptibility gene for high-grade myopia, a selection is essential of a patient group demonstrating a more pronounced impact from genetic factors whilst reducing to a minimum any possible impact from environmental factors. For this reason we imposed a very rigid criteria for the selection of the high myopia group patients. The mandatory entry criteria in our study were patients with very severe myopia of \( \geq -9.25 \) diopters for both eyes, ocular axial lengths of \( \geq 26 \) mm for both eyes, and devoid of any ocular diseases, such as staphyloma on funduscopy. Therefore, we are of the view that the myopic individuals in our patient group were narrowed down to high myopes with a greater impact from genetic factors.

In 1998, Young et al (1988b) performed a linkage analysis of eight families with high myopia in two or three successive generations, containing a total of 82 individuals to obtain the maximum lod score of 9.59 for the microsatellite marker D18S481. This region, which mapped at 7.6 cM on the short arm of chromosome 18, was indicated to be a susceptibility genetic locus for high myopia. Although there are several genes already identified in this region (Scavello et al 2005), of these, due to laminin being a major constituent of the sclera and the laminin \( \alpha 1 \) (\textit{LAMA1}) gene encoding the A chain of laminin being neighboured in this MYP2 region, \textit{LAMA1}...
### Table 3 Frequency analysis of the estimated haplotype for the SNPs within the LAMA1 gene in Japanese myopia patients and control subjects

| Haplotype | SNPs | 334384 | 334420 | 334407 | 12454984 | 684634 | 12961939 | 12607841 | 1164063 | 671871 | 61950868 | 2013077 |
|-----------|------|--------|--------|--------|----------|--------|----------|----------|--------|--------|----------|--------|
|           | Case | Freq.  | OR(95%CI) | p value |
| 01        | C    | 0.157  | 0.88(0.58–1.34) | 0.293 |
| 02        | T    | 0.141  | 1.40(0.87–2.24) | 0.096 |
| 03        | T    | 0.070  | 1.04(0.57–1.91) | 0.299 |
| 04        | T    | 0.052  | 2.46(1.01–5.97) | 0.010 ** |
| 05        | C    | 0.044  | 1.60(0.68–3.73) | 0.102 |
| 06        | C    | 0.028  | 1.05(0.41–2.71) | 0.408 |
| 07        | C    | 0.023  | 1.14(0.40–3.25) | 0.396 |
| 08        | T    | 0.021  | 1.44(0.44–4.72) | 0.274 |
| 09        | T    | 0.020  | 0.75(0.26–2.12) | 0.299 |
| 10        | T    | 0.018  | 1.47(0.40–5.35) | 0.377 |

*Odds ratio (OR) of estimated haplotypes are indicated. OR is calculated by Woolf’s formular using estimated frequencies.
P value (P < 0.05) is significant by a χ² test.
**This value is not significant after Bonferroni correction.
attracted attention as the promising candidate gene for high myopia (Young, Ronan, Drahozal et al 1998).

An increase in the ocular axial length is observed in individuals with high-degree myopia. Although the exact mechanism underlying this axial length elongation has yet to be defined, it is presumed that a blurred image or light projected onto the retina induces secretion of some substance from cells, such as the visual, amacrine, horizontal and bipolar cells, which evokes transfer of the signal to the sclera, thus leading to scleral remodeling. Therefore, the possibility is inferred that genetic polymorphisms (individual differences) exist in such genes as laminin and collagen IV, which are scleral components. In addition, these individual differences between the same genes could bring about individual differences in scleral extension and remodelling, leading to the situation where there are individuals who become myopic and those who do not, despite being in similar living environments. To our knowledge, SNP analysis of the LAMA1 gene in patients with high myopia has not been previously conducted and that our study is the first large-scale correlative analysis of the LAMA1 gene in subjects with severe myopia. Although we analyzed SNPs located on the LAMA1 gene in myopic subjects with a severe degree of myopia by imposing very rigid selection criteria, none of the SNPs exhibited a statistically significant increment or decrement in the patient groups analyzed. Analysis of each allele at each SNP using both dominant and recessive models of their respective alleles also revealed that none of the SNPs displayed statistically significant between-group differences.

In this study, we selected a total of 13 SNPs of the LAMA1 gene ensuring their wide distribution over the regions ranging from exon 1 to 62 with a view to conducting an extensive analysis of the LAMA1 gene that consists of 62 exons and occupies 176 kb within the genome. As 13 SNPs are located at appropriate intervals in the LAMA1 gene, other polymorphisms in the LAMA1 gene may not be associated with high myopia like 13 SNPs. However, to take the narrow ranged LD blocks in the LAMA1 gene into consideration, it is suggested that the results of 13 SNPs in this study don’t necessarily reflect the relations between high myopia and all polymorphisms in the LAMA1 gene. Thus, more detailed SNPs analysis in the LAMA1 gene may be necessary for the complete screen of the entire LAMA1 genes.

In conclusion, there were no statistically appreciable differences between the high myopia group and the control group for the 13 SNPs analyzed in the LAMA1 gene encoding the chain A of laminin. Therefore, the possibility of the LAMA1 gene being primarily associated with the pathogenesis of high myopia is deemed low. In the future, we would like to further our analyses of other genes as well as those that are present within the MYP2 region.
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