Discussion

The present study found a significant association between the SERPINF1 gene variants and the clinical response of PCV to PDT; those patients who were homozygous for the minor allele A of SERPINF1 rs12603825 were administered an additional treatment within a significantly shorter period of time after the first PDT, were significantly less apt to be inactivated by a single treatment (independently of baseline clinical characteristics and smoking status), and had significantly worse visual acuity after PDT than those with no more than 1 copy of the minor allele.

SERPINF1 gene encodes serpin peptidase inhibitor, clade F, member 1, which is also referred to as pigment epithelium-derived factor (PEDF), and was purified first from conditioned medium of human retinal pigment epithelial cells as a factor with potent neural differentiating activity.[27] Subsequent studies have revealed significantly reduced expression of PEDF in retinal pigment epithelial cells, Bruch's membrane,[28,29] and the vitreous[30] of eyes with AMD, whereas other studies have demonstrated the impact of PDT on the expression of PEDF.[31–33] By taking into consideration that PEDF inhibits the migration of endothelial cells in vitro and the in vivo development of experimental retinal neovascularization and choroidal

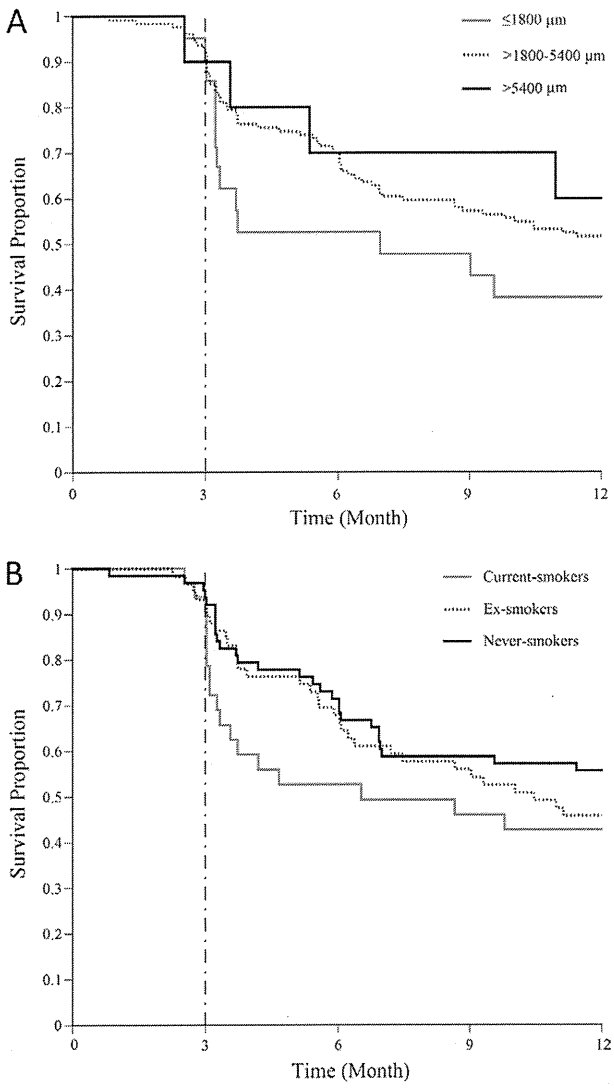

Figure 3. Overall survival analysis curve for the retreatment-free period by the 3 groups of GLD (**A**) and smoking status (**B**). There was no significant difference among these groups (P = 0.214 and 0.166, respectively), although borderline evidence of an association was observed between those who never smoked and ex-smokers plus current smokers (P = 0.060).

Table 4. Clinical Characteristics and Genotype Distribution of the Study Population by Response to Single Photodynamic Therapy

	Photodynamic Therapy Less Effective*	Photodynamic Therapy Effective*	Adjusted P Value†
No. of patients	13	150	
Mean age (yrs)	69.92	72.19	0.222
Gender			0.283
Women	4 (30.8)	42 (28.0)	
Men	9 (69.2)	108 (72.0)	
Smoking history			0.489
Never	4 (36.4)	59 (41.3)	
Previous	4 (36.4)	55 (38.5)	
Current	3 (27.2)	29 (20.3)	
GLD			0.677
≤1800 μm	2 (15.4)	18 (12.4)	
1800–5400 μm	10 (76.9)	118 (81.4)	
>5400 μm	1 (7.7)	9 (6.2)	
SERPINF1_rs12603825		(GA+GG) vs. AA	0.0027
AA	4 (30.7)	10 (6.8)	
GA	2 (15.4)	60 (40.5)	
GG	7 (53.8)	78 (52.7)	

GLD = greatest linear dimension.
*Patients were divided into 2 subgroups by whether additional treatment was required within the first 3-month follow-up after a single PDT. Less effective = required; effective = not required.
†Adjusted for age, gender, smoking status, greatest linear dimension, and genotype.

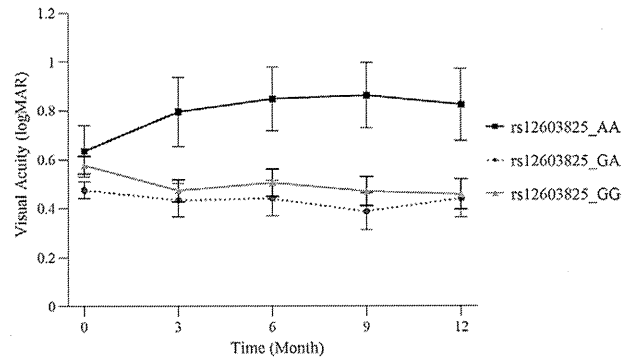

Figure 6. Visual prognosis by SERPINF1 rs12603825 after PDT. Visual outcomes of patients with the AA genotype were significantly worse than those with other genotypes (P = 0.013). Error bars represent ± 1 standard error of the mean. logMAR = logarithm of the minimum angle of resolution.

neovascularization,[34–36] the findings are consistent with those of the present study showing an association between the PEDF gene variants and the response to PDT.

The present study also shows the possibility that PEDF polymorphisms affect PEDF expression in eyes with PCV. We then evaluated whether rs12603825 affects PEDF gene expression in vivo using the raw data deposited in the Gene Expression Omnibus[37] as GSE 6536 (available at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6536, accessed July 1, 2010). However, there proved to be no association between SERPINF1 rs12603825 and PEDF gene expression ($P = 0.689$, analysis of variance test), and no significant differences in the baseline clinical characteristics among various genotypes of this SNP (Table 5, available at http://aaojournal.org.). Thus, the PEDF polymorphism may not result in the phenotypic difference via a change in PEDF gene expression. PEDF polymorphisms may influence the binding affinity to the receptor or indirectly affect PEDF expression after PDT by affecting the pathway between PDT and the PDT-induced change of PEDF expression.

As shown by the patients with rs12603825 AA genotype (Fig 1), another possibility regards PEDF polymorphisms as determinants of the probability of hemorrhage after PDT in eyes with PCV, which influences visual prognosis in the long term. All 3 patients with a macular hemorrhage among those who required additional treatment within 3 months after PDT had an rs12603825 AA genotype. Recurrent hemorrhage is one of the most symbolic signs of PCV,[19,21] and visual outcome is poor in eyes that have a massive subretinal hemorrhage involving the macula.[14,38] Furthermore, subretinal hemorrhage after PDT is a common finding in patients with PCV.[39,40] Yokoi et al[41] reported that PEDF levels in vitreous fluid were associated with vitreous hemorrhage in proliferative diabetic retinopathy, but the relationship between hemorrhage and PEDF is not fully understood. With this hypothesis in mind, our study may enhance our understanding of the mechanisms of hemorrhage in PCV.

Previous studies have shown the possibility that small lesions in patients with AMD respond better to PDT than larger lesions,[10,42] but the current study found no significant association between baseline lesion size and response to PDT. Conversely, we found that individuals who never smoked were less prone to recurrence than ex-smokers or current smokers. This result seems to be in agreement with the numerous studies that have shown smoking to be a risk factor for the development of AMD[43–46] and that smoking strongly influences the development of PCV.[18–20]

Study Limitations

One limitation of the present study is the number of participants. We found no significant association between rs12603825 and the retreatment-free period in eyes with PCV that responded to a single administration of PDT, although patients homozygous for the minor allele did tend to be administered additional treatment within a shorter period of time than those with other genotypes in the long term (Fig 4, available at http://aaojournal.org.). This tendency might reach statistical significance if the number of patients were increased. Other reports have demonstrated an association between the initial response and the risk of recurrence in other diseases.[47,48] Another limitation is the subgroup that initially responded to PDT may not represent a true difference in histologic response to PDT, because this relied on clinical information. Further basic research is needed to better characterize the relationship between the PEDF gene and the response to PDT. Another limitation is the absence of evaluation for the response to repeated treatments of PDT. Approximately half of the patients who noted less-effective responses to the first PDT received other treatments (e.g., anti-VEGF therapy or combined treatment) as their additional therapy. Further validation studies (e.g., prospective study) are obviously needed to clarify the detailed clinical response to PDT.

In conclusion, this study provides the first evidence that clinical, environmental, and genetic factors influence the response of PCV to PDT: PEDF gene variants associate independently with their response to PDT. Although it remains controversial as to whether PCV represents a subtype of neovascular AMD, the response to PDT is completely different for PCV and for neovascular AMD. Intravitreal injection of adenoviral vectors containing PEDF complementary DNA has been suggested to be a viable approach to therapy for neovascular AMD;[49,50] thus, our findings may lead to ways to modify the effects of PDT, to new methods of treatment using these materials, and to an understanding of the pathogenesis of PCV.

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Footnotes and Financial Disclosures

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Genetics

Association of 15q14 and 15q25 with High Myopia in Japanese

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Purpose. To investigate whether there are associations of genetic variations in chromosome 15q14 and 15q25, recently shown to confer risk of refractive error and myopia in Caucasians, with high myopia in Japanese.

Methods. A total of 1125 unrelated Japanese patients with high myopia and two independent control groups were evaluated (366 cataract patients without high myopia and 929 healthy Japanese patients). The four single nucleotide polymorphisms (SNPs) rs634990 and rs524952 at 15q14 and rs8027411 and rs17175798 at 15q25 were genotyped.

Results. A significant association with high myopia was observed in 15q14 ($P = 0.0035$ for rs634990 and $P = 0.0017$ for rs524952 when evaluated with cataract controls and $P = 1.91 \times 10^{-6}$ for rs634990 and $P = 8.78 \times 10^{-7}$ for rs524952 with healthy Japanese controls). When evaluated with cataract controls, the odds ratios (95% confidence intervals) were 1.30 (1.10–1.53) for rs634990 C allele and 1.32 (1.11–1.56) for rs524952 A allele. The population attributable risks were 0.29 and 0.30, respectively. The SNPs in 15q25 did not show a significant association with high myopia when evaluated with cataract control ($P > 0.42$), while it showed a weak association when evaluated with healthy Japanese controls ($P = 0.031$ for rs8027411 and $P = 0.047$ for rs17175798) with odds ratios of 1.17 (1.03–1.33) for rs8027411 T allele and 1.15 (1.02–1.31) for rs17175798 C allele.

Conclusions. These findings suggest that a region in 15q14 is susceptibility loci for high myopia. This locus harbor susceptibility genes for not only common myopia but also for high myopia. The 15q25 locus might also have association to myopia. (*Invest Ophthalmol Vis Sci.* 2011;52:4853–4858) DOI:10.1167/iovs.11-7311

Myopia is the most common visual disorder in the world, with the prevalence estimated to be 25% in the United States and Western Europe and to be much higher (40%–70%) in Asians, including Chinese and Japanese.[1–5] Myopic eyes with very long axial lengths (≥26 mm) or a high degree of myopic refractive error (≤ −6 D) are classified as high myopia.[6] Recently, the prevalence of high myopia has been increasing worldwide, especially in the younger East Asian population.[7,8] It is well known that high myopia is associated with various ocular complications,[9] and it is one of the leading causes of legal blindness in developed countries.[10–12] Therefore, it is important to develop methods for preventing or delaying the onset of high myopia or for limiting its progression.

The cause of myopia is not fully understood, and, in fact, it is not yet clear that common myopia and high myopia share the same background; high myopia may have a unique background that distinguishes it from common myopia. It has been shown that environmental factors (in particular near work, a higher level of education, and less time spent in outdoor activities) and genetic factors contribute to the development of myopia.[13] Twin studies provide compelling evidence that myopia is inherited,[14,15] and multiple family-based whole genome linkage analysis indicate several heritable myopia susceptibility loci, such as the MYP1–18 loci.[16–31] Some of these loci are reported to be associated with common myopia, high myopia, or both. However, several studies could not replicate these associations.[32–34] In addition, many candidate genes associated with high myopia have been reported.[35–43] However, most of these associated candidate genes have been negated by subsequent studies, and no genes have yet been identified that are consistently responsible for either common or high myopia.

Genome-wide association studies (GWAS) are expected to reveal the susceptibility genes of many complex diseases, as shown in age-related macular degeneration (AMD). We performed the first genome-wide association study for high myopia and showed that several single nucleotide polymorphisms (SNPs) at 11q24.1 are associated significantly with high myopia in Japanese.[44] However, a study in Han Chinese did not show the association of rs577948, one of the SNPs, with high myopia.[45]

Recently, two other groups have performed GWAS for refractive error and showed that SNPs in 15q14 and 15q25 are associated with refractive error and myopia,[46,47] but the cohorts used in these two studies were population-based, and only a limited number (1.7%–4.0%) of patients with high myopia were included. Although it is not clear if common and high myopia share the same genetic background, some MYP loci are reported to be associated with both common myopia

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and high myopia. In this study, we evaluated the associations of reported SNPs with high myopia in Japanese.

Moreover, we investigated their associations with the occurrence of choroidal neovascularization (CNV) in high myopia. Some highly myopic eyes develop CNV, while other highly myopic eyes do not, and because CNV is one of the most vision-threatening complications in highly myopic eyes, the investigation of the mechanisms of how it occurs is important. We evaluated the influence of susceptibility genes for neovascular AMD on the occurrence of CNV in myopic eyes and found that these genes do not affect the development of myopic CNV.[48] Axial elongation of highly myopic eyes results in thinning of the retina and choroid, patchy chorioretinal atrophy, and lacquer cracks, all of which are important predisposing conditions for the development of CNV.[49,50] It could be hypothesized that CNV occurs when the eye is strongly affected by susceptibility genes for myopia, or it may be that specific susceptibility genes exist for myopic CNV—genes that are in addition to the susceptibility genes for myopia.

Materials and Methods

All procedures in this study adhered to the tenets of the Declaration of Helsinki. The institutional review boards and the ethics committees of each institution involved approved the protocols of this study. All patients were fully informed of the purpose and procedures of this study, and written consent was obtained from each patients.

Patients

One thousand one hundred twenty-five unrelated highly myopic Japanese patients were recruited from Kyoto University Hospital, Tokyo Medical and Dental University Hospital, Ozaki Eye Hospital, and Otsu Red-cross Hospital (mean age ± SD, 57.6 ± 14.8 years; male/female ratio, 33.5%/66.5%). All patients had a comprehensive ophthalmic examination, including dilated indirect and contact lens slit lamp biomicroscopy, automatic objective refraction, and measurements of the axial length by applanation A-scan ultrasonography or partial coherence interferometry (IOLMaster; Carl Zeiss Meditec, Dublin, CA). The difference between these two devices would be around 0.1 mm.[52] When a patient who visited the aforementioned hospitals had an axial length of >26.1 mm in both eyes, peripheral blood was obtained after informed consent and used as high-myopia patients group. To evaluate the contribution of the SNPs between high myopic patients with CNV and high myopic patients without CNV, the high myopic patients group was separated into a CNV group (600 patients) and a no CNV group (450 patients). Inclusion criteria of the CNV group were clinical presentation and angiographic manifestations of macular CNV or Fuchs' spot in at least one eye.

As the control subjects, we used 366 cataract patients with axial lengths <25.0 mm in both eyes (control 1). These patients were recruited from the Department of Ophthalmology at Kyoto University Hospital, the Ozaki Eye Hospital, the Japanese Red Cross Otsu Hospital, and Nagahama City Hospital; their mean age (± SD) was 74.4 ± 8.4 years, and there were 39.9% men and 60.1% women. The axial length was measured with applanation A-scan ultrasonography or partial coherence interferometry before cataract surgery and dilated fundus examination was performed after surgery. If the fundus examination revealed myopic change such as lacquer cracks/peripapillary atrophy, staphyloma or CNV, the subject was eliminated from the control 1 group.

We also used DNA samples from 929 subjects who were randomly selected from the Pharma SNP Consortium (PSC); this constituted control group 2. This group has been used for previous genomic studies and is regarded as being representative of the general Japanese population (mean age ± SD, 38.8 ± 11.8 years; male/female ratio, 61.7%/38.3%).[51] All were Japanese and none had any history of ocular disease.

Genotyping and Statistical Analyses

Genomic DNAs were prepared from peripheral blood using a DNA extraction kit (QuickGene-610L; Fujifilm, Minato, Tokyo, Japan). Four SNPs (rs634990, rs524952, rs8027411, and rs17175798) were selected based on their specific presence in two previous GWAS; they were genotyped using a commercially available assay (TaqMan SNP assay with the ABI PRISM 7700 system; Applied Biosystems, Foster City, CA). Deviations in genotype distributions from the Hardy-Weinberg equilibrium (HWE) were assessed for each group with the HWE exact test. The χ^2 test for trend or its exact counterpart was used to compare the genotype distributions of two groups. To adjust age and sex, we performed multiple regression and logistic regression analysis. These statistical analyses were performed with R software R (R Foundation for Statistical Computing, Vienna, Austria, available at http://www.rproject.org/). $P < 0.05$ was considered statistically significant.

Results

The demographics of the study population are shown in Table 1. The axial length of the 2258 eyes of the 1129 high myopia cases ranged from 26.11 to 39.73 mm, with a mean ± SD of

Table 1. Characteristics of the Study Population

	Patients	Controls	
	High Myopia*	Cataract†	PSC‡
Patients, *n*	1125	366	929
Age in years, Mean ± SD	57.57 ± 14.75	74.40 ± 8.37	38.81 ± 11.83
Sex, *n* (%)			
Male	377 (33.5%)	146 (39.9%)	573 (61.7%)
Female	748 (66.5%)	220 (60.1%)	356 (38.3%)
Axial length, mm ± SD			
Right eyes	29.18 ± 1.95	23.05 ± 1.00	NA
Left eyes	29.01 ± 2.16	23.01 ± 1.66	NA
Refraction of the phakic eyes, D§			
Right eyes	−10.79 ± 6.96	−0.23 ± 2.56	NA
Left eyes	−10.36 ± 5.82	−0.11 ± 2.52	NA

* Axial length of >26.1 mm in both eyes.

† Individuals who underwent cataract surgery and who had an axial length <25.00 mm in both eyes.

‡ DNA samples randomly selected from the Pharma SNP Consortium (PSC).

§ For calculations of refraction, eyes that had undergone cataract surgery or corneal refractive surgery were excluded.

TABLE 2. Genotype Counts, Associations, and Odds Ratios in the High Myopia Patients and Cataract Controls

Locus	SNP ID	Genotype	High Myopia	Controls	*P**	Adjusted *P*†	OR (95% CI)	PAR
15q14	rs634990 (C/T)	CC	304	84	0.0026	0.0035	1.65 (1.19-2.29)	0.29
		CT	571	165			1.58 (1.19-2.09)	
		TT	246	112			1.00 (ref)	
	rs524952 (A/T)	AA	303	81	0.0015	0.0017	1.70 (1.22-2.37)	0.30
		AT	572	164			1.59 (1.19-2.11)	
		TT	244	111			1.00 (ref)	
15q25	rs8027411 (G/T)	TT	428	116	0.17	0.42	0.87 (0.60-1.26)	0.03
		GT	525	193			1.17 (0.82-1.67)	
		GG	166	52			1.00 (ref)	
	rs17175798 (C/T)	CC	422	117	0.25	0.60	1.12 (0.77-1.62)	0.04
		CT	528	193			0.85 (0.60-1.20)	
		TT	171	53			1.00 (ref)	

SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval; PAR, population attributable risk.
* Differences in the observed genotypic distribution were examined by χ^2 test for trend.
† Age and sex adjustment was performed based on a logistic regression model.

29.09 ± 2.04 mm. Among the 2250 eyes enrolled, 1753 (77.9%) were phakic, 427 (19.0%) were pseudophakic, and 70 (3.1%) were aphakic. The mean refraction of the 1753 phakic eyes was −10.52 ± 6.71 D. The axial length of the 732 eyes of the cataract patients ranged from 18.67 to 24.89 mm, with a mean ± SD of 23.02 ± 1.28 mm. Mean refraction of the phakic eyes in control group 1 was −0.14 ± 2.2 D.

Genotype counts, associations examined with χ^2 test for trend, and odds ratios for the four SNPs between the high myopia patients and the controls are shown in Tables 2 and 3. The distributions of the genotypes for the four SNPs were all in HWE ($P > 0.1$), as assessed with the exact test. Rs634990 and rs524952 were in almost complete linkage disequilibrium, as were rs8027411 and rs17175798 ($D' > 0.95$).

When evaluated with control group 1 (cataract patient controls, $n = 366$), the SNPs rs634990 and rs524952 in 15q14 showed a significant association with high myopia ($P = 0.0035$ and 0.0017, respectively). The odds ratios (95% confidence intervals [CIs]) were 1.30 (1.10-1.53) for rs634990 C allele and 1.32 (1.11-1.56) for rs524952 A allele. When evaluated with control group 2 (healthy Japanese controls, $n = 929$), these two SNPs showed strong association with high myopia ($P = 2.21 \times 10^{-6}$ and 1.62×10^{-6}, respectively). The odds ratios were 1.36 (1.20-1.54) for rs634990 C allele and 1.37 (1.21-1.55) for rs524952 A allele. The population attributable risk (PAR) was 0.29 for rs634990 and 0.30 for rs524952 when evaluated with both controls, and the risk alleles were the same as those reported previously.

The variants in 15q25 (rs8027411 and rs17175798) showed no association to high myopia when evaluated with control group 1 ($n = 366$), while these two SNPs showed marginal associations ($P = 0.031$ and 0.047, respectively) when evaluated with control group 2 ($n = 929$). The minor allele frequencies in these two controls were very similar; 0.41 for both SNPs in control group 1 and 0.42 for both SNPs in control group 2, and the risk alleles seemed to be the same as those reported previously. The odds ratios were 1.13 (0.95-1.34) for rs8027411 T allele and 1.10 (0.93-1.31) for rs17175798 C allele when evaluated with control group 1 and 1.17 (1.03-1.33) and 1.15 (1.02-1.31), respectively, when evaluated with control group 2. The PARs were 0.03 for rs8027411 and 0.04 for rs17175798 when evaluated with control group 1 and 0.17 and 0.14, respectively, when evaluated with control group 2.

Associations for the four SNPs with CNV in highly myopic eyes of the Japanese population in this study are shown in Table 4. We analyzed the genotype distributions for rs634990, rs524952, rs8027411, and rs17175798 in high myopia patients with CNV and compared them with high myopia patients

TABLE 3. Genotype Counts, Associations, and Odds Ratios in the High Myopia Patients and Pharma SNP Consortium Controls

Locus	SNP ID	Genotype	High Myopia	Controls	*P**	Adjusted *P*†	OR (95% CI)	PAR
15q14	rs634990 (C/T)	CC	304	191	1.1×10^{-6}	1.91×10^{-6}	1.84 (1.44-2.36)	0.29
		CT	571	442			1.50 (1.21-1.85)	
		TT	246	285			1.00 (ref)	
	rs524952 (A/T)	AA	303	191	7.60×10^{-7}	8.78×10^{-7}	1.86 (1.45-2.39)	0.30
		AT	572	444			1.51 (1.22-1.86)	
		TT	244	286			1.00 (ref)	
15q25	rs8027411 (G/T)	TT	428	310	0.013	0.031	1.37 (1.06-1.78)	0.17
		GT	525	445			1.17 (0.91-1.51)	
		GG	166	165			1.00 (ref)	
	rs17175798 (C/T)	CC	422	309	0.026	0.047	1.32 (1.02-1.71)	0.14
		CT	528	453			1.12 (0.88-1.44)	
		TT	171	165			1.00 (ref)	

SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval; PAR, population attributable risk.
* Differences in the observed genotypic distribution were examined by χ^2 test for trend.
† Age and sex adjustment was performed based on a logistic regression model.

TABLE 4. Associations of the Four Single Nucleotide Polymorphisms with Choroidal Neovascularization Development in Highly Myopic Eyes

	15q14						15q25					
	rs634990 C			rs524952 A			rs8027411 T			rs17175798 C		
	Frq	OR (95% CI)	P*	Frq	OR (95% CI)	P*	Frq	OR (95% CI)	P*	Frq	OR (95% CI)	P*
High myopia with no CNV (n = 600)	0.53			0.53			0.61			0.61		
High myopia with CNV (n = 450)	0.51	0.93 (0.78-1.11)	0.41	0.51	0.93 (0.78-1.10)	0.38	0.62	1.04 (0.87-1.25)	0.64	0.60	0.95 (0.79-1.13)	0.54

Frq, allele frequency.

* Age and sex adjustment was performed based on a logistic regression model.

without CNV to check for a possible difference between these two groups in the genetic background of the four myopia-susceptibility variants. Distributions of these four genotypes were all in HWE ($P > 0.2$), and there was no significant difference in the allele frequencies of SNPs between high myopia with CNV and high myopia without CNV—even after age and sex adjustment.

The average age was 54.9 ± 14.9 years in the patients without CNV, while the average age was 60.7 ± 13.3 years in the patients with CNV ($P < 0.0001$). Because the average age was significantly higher in the group with CNV, we performed subanalysis dividing the cohort into 40- to 49-year-old, 50- to 59-year-old, 60- to 69-year-old, and 70- to 79-year-old subgroups (Table 5). Our subanalysis revealed that there were no associations between CNV occurrence and the genotype variation in rs634990, rs524952, rs8027411, and rs17175798.

DISCUSSION

Myopia has been thought to be a multifactorial disease, and for more than a decade many researchers have sought the susceptibility genes associated with myopia. Several chromosome loci have been reported to be associated with common myopia, high myopia, or both[16-31]; however, some other investigators could not replicate these associations.[32-34] To date, no genes have been identified that are consistently responsible for either common or high myopia. Furthermore, it has not been clear if common and high myopia share the same genetic background or if high myopia has a unique genetic background that distinguishes it from common myopia. In the present study, we have shown that SNPs in 15q14 are associated significantly with high myopia in Japanese and that SNPs in 15q25 might also be associated. These same SNPs have been reported recently to be associated with myopia in Caucasians,[46,47] although the Caucasian cohort was population-based and patients with high myopia were extremely rare (1.7%-4.0%). Common and high myopia may well share the same genetic background—at least in part.

In the present study, we used two distinct control groups: cataract patients with axial lengths <25.00 mm in both eyes (control group 1, n = 366) and representative of the general Japanese population (control group 2, n = 929). Because control group 2 is representative of the general Japanese population, high myopia patients may well be included as control subjects. When one considers the high rate of high myopia in Japanese compared to Caucasians, in whom it is estimated to be present in approximately 5% of the general population, this could weaken the detection of any genetic association with high myopia. However, we believe that using the general population as a control is certainly acceptable for a case control study of high myopia.[44]

When evaluated with both controls, 15q14 showed a significant association with high myopia. The odds ratio of rs634990 CC genotype to TT genotype was 1.65 (95% CI, 1.19-2.29) when compared with control group 1 and 1.84 (95% CI, 1.44-2.36) when compared with control group 2, findings that are compatible with the reported odds ratio of 1.83 (1.42-2.36) for refractive error and myopia in a general population of Caucasians. The genetic variation at 15q14 seems to contribute to common myopia and to high myopia in a similar manner.

With 15q25, however, the association significance was only marginal when evaluated with control group 2, and the analysis with control group 1 showed no association to high myopia. In accordance with the original report, the risk allele was T in rs8027411. Because control group 2 consisted of the general population, and because patients with high myopia may be included as control subjects, this could lower the power of this study to detect a genetic association with high myopia. However, two SNPs in 15q25 were significantly associated with high myopia evaluated with control group 2. Considering that the minor allele frequencies in 15q25 were very similar be-

TABLE 5. Subanalysis of the Associations of the Four Single Nucleotide Polymorphisms with Choroidal Neovascularization Development in Highly Myopic Eyes

	n		P Value*			
Age, y	High Myopia with CNV	High Myopia with No CNV	rs634990	rs524952	rs8027411	rs17175798
<40	37	99	0.55	0.42	0.63	0.63
40-49	47	92	0.13	0.14	0.18	0.28
50-59	91	143	0.88	0.89	0.74	0.79
60-69	145	149	0.79	0.73	0.98	0.66
70-79	103	84	0.91	0.90	0.48	0.61
≥80	27	33	0.58	0.61	0.54	0.65

tween our control groups 1 (n = 366) and 2 (n = 929) and the original study showed that the odds ratio was very low as 1.16 (1.02–1.28) for the rs8027411 T allele, the size of control group 1 might be insufficient to detect the association. Even for the analysis with control group 2, the association becomes negative after Bonferroni correction. We might have to negate the association of 15q25 locus. Although our present study could be regarded as a replication study for the association of previously reported loci to myopia, and because the Bonferroni correction might not be applicable for a replication study, we would have to interpret the association of 15q25 locus in the present study with caution. Because the association of 15q25 to myopia was reported to be very low and the minor allele frequencies in 15q25 were very similar between control groups 1 and 2, additional study with a larger cohort might reveal the true association of 15q25 to myopia.

We also evaluated the genetic difference between high myopia patients with CNV and high myopia patients without CNV, and found that the genotype distribution of the SNPs evaluated was not significantly different. These high myopia patients without CNV might develop CNV later. Because CNV will develop only in 5% to 10% of high myopia patients and because it typically starts to develop in the fourth or fifth decades of life, the number of patients who will develop CNV later in these groups would be limited. However, the individuals who will develop CNV later in the control cohort would weaken the power to detect the associations to CNV development. Our negative result might be partly related to this weakened power. To eliminate the influence of such individuals, we performed subanalysis dividing the cohort into 40- to 49-year-old, 50- to 59-year-old, 60- to 69-year-old, and 70- to 79-year-old subgroups. Our subanalysis showed no associations of these loci to the CNV development. Although our findings should be interpreted with caution, factors other than 15q14 and 15q25 might affect the development of CNV in highly myopic eyes. Lacquer cracks and peripapillary atrophy can be the basis of CNV development. Although lacquer cracks and peripapillary atrophy do not always lead to CNV development, genomic studies paying attention to these features might give us loci associated with CNV development. The occurrence of CNV beneath the fovea is one of the most vision-threatening complications of highly myopic eyes, so it is important to study the mechanisms of CNV occurrence in such eyes even after susceptibility genes for myopia are known.

Because genetic associations have ethnic differences, our findings could not be compared directly to the previous studies that were performed in Caucasians. Furthermore, these previous reports of associations of 15q14 and 15q25 with myopia used a general population, while we performed a case control study using high myopia patients as the cases and the general population as controls, so our control cohort is almost the same as the entire cohort used in the previous studies. Although the associations of 15q14 and 15q25 to refractive error (myopia) and/or high myopia need to be evaluated in various ethnicities, our findings of similar contribution of these loci to common myopia in Caucasians and to high myopia in Japanese suggests that 15q14 and 15q25 contribute to these two conditions in a similar manner, although additional studies might reveal genetic differences that differentiate high myopia from common myopia. Moreover, additional genetic background might also affect the occurrence of myopia/high myopia, given at most 30% of PAR.

In conclusion, we have shown that genetic variations at 15q14 are associated significantly with high myopia in Japanese. Based on our findings and those of previous studies, this might be a susceptibility locus for both myopia and high myopia. The association of 15q25 should be evaluated in additional studies with larger cohorts. Our findings also suggest that CNV occurs independent of genetic variations at these loci, and that other factors affect the occurrence of CNV in highly myopic eyes.

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The Progression of Liver Fibrosis Is Related with Overexpression of the miR-199 and 200 Families

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Abstract

Background: Chronic hepatitis C (CH) can develop into liver cirrhosis (LC) and hepatocellular carcinoma (HCC). Liver fibrosis and HCC development are strongly correlated, but there is no effective treatment against fibrosis because the critical mechanism of progression of liver fibrosis is not fully understood. microRNAs (miRNAs) are now essential to the molecular mechanisms of several biological processes. In order to clarify how the aberrant expression of miRNAs participates in development of the liver fibrosis, we analyzed the liver fibrosis in mouse liver fibrosis model and human clinical samples.

Methodology: In a CCL_4-induced mouse liver fibrosis model, we compared the miRNA expression profile from CCL_4 and olive oil administrated liver specimens on 4, 6, and 8 weeks. We also measured expression profiles of human miRNAs in the liver biopsy specimens from 105 CH type C patients without a history of anti-viral therapy.

Principle Findings: Eleven mouse miRNAs were significantly elevated in progressed liver fibrosis relative to control. By using a large amount of human material in CH analysis, we determined the miRNA expression pattern according to the grade of liver fibrosis. We detected several human miRNAs whose expression levels were correlated with the degree of progression of liver fibrosis. In both the mouse and human studies, the expression levels of miR-199a, 199a*, 200a, and 200b were positively and significantly correlated to the progressed liver fibrosis. The expression level of fibrosis related genes in hepatic stellate cells (HSC), were significantly increased by overexpression of these miRNAs.

Conclusion: Four miRNAs are tightly related to the grade of liver fibrosis in both human and mouse was shown. This information may uncover the critical mechanism of progression of liver fibrosis. miRNA expression profiling has potential for diagnostic and therapeutic applications.

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Introduction

Chronic viral hepatitis is a major risk factor for hepatocellular carcinoma (HCC) [1]. Worldwide 120–170 million persons are currently chronically Hepatitis C Virus (HCV) infected [2]. Due to repetitive and continuous inflammation, these patients are at increased risk of developing cirrhosis, subsequent liver decompensation and/or hepatocellular carcinoma. However, the current standard of care; pegylated interferon and rivabirin combination therapy is unsatisfied in the patients with high titre of HCVRNA and genotype 1b. Activated human liver stellate cells (HSC) with chronic viral infection, can play a pivotal role in the progression of liver fibrosis [3]. Activated HSC produce a number of profibrotic cytokines and growth factors that perpetuate the fibrotic process through paracrine and autocrine effects.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that control gene expression by degrading target mRNA or suppressing their translation [4]. There are currently 940 identifiable human miRNAs (The miRBase Sequence Database - Release ver. 15.0). miRNAs can recognize hundreds of target genes with incomplete complementary; over one third of human genes appear to be conserved miRNA targets [5][6]. miRNA is associated several pathophysiologic events as well as fundamental cellular processes such as cell proliferation and differentiation. Aberrant expression of miRNA can be associated with the liver diseases [7][8][9][10]. Recently reported miRNAs can regulate the activation of HSCs and thereby regulate liver fibrosis. miR-29b, a negative regulator for the type I collagen and SP1, is a key regulator of liver fibrosis [11]. miR-27a and 27b allowed culture-activated rat HSCs to switch to a more quiescent HSC phenotype,

with restored cytoplasmic lipid droplets and decreased cell proliferation [12].

In this study, we aimed to reveal the association between miRNA expression patterns and the progression of liver fibrosis by using a chronic liver inflammation model in mouse. We also sought to identify the miRNA expression profile in chronic hepatitis (CH) C patients according to the degree of liver fibrosis, and to clarify how miRNAs contribute to the progression of liver fibrosis. We observed a characteristic miRNA expression profile common to both human liver biopsy specimens and mouse CCL_4 specimens, comprising the key miRNAs which are associated with the liver fibrosis. This information is expected to uncover the mechanism of liver fibrosis and to provide a clearer biomarker for diagnosis of liver fibrosis as well as to aid in the development of more effective and safer therapeutic strategies for liver fibrosis.

Results

The expression level of several mouse miRNAs was increased by introducing mouse liver fibrosis

In order to identify changes in the miRNA expression profile between advanced liver fibrosis and non-fibrotic liver, we intraperitoneally administered CCL_4 in olive oil or olive oil alone twice a week for 4 weeks and then once a week for the next 4 weeks. Mice were sacrificed at 4, 6, or 8 weeks and then the degree of mouse liver fibrosis was determined by microscopy (Figure S1). miRNA expression analysis was performed from the liver tissue collected at the same time. Histological examination revealed that the degree of liver fibrosis progressed in mice that received CCL_4 relative to mice receiving olive oil alone (Figure 1A). Microarray analysis revealed that in CCL_4 mice, the expression level of 11 miRNAs was consistently higher than that in control mice (Figure 1B).

miRNA expression profile in each human liver fibrosis grade

We then established human miRNAs expression profile by using 105 fresh-frozen human chronic hepatitis (CH) C liver tissues without a history of anti-viral therapy, classified according to the grade of the liver fibrosis (F0, F1, F2, and F3 referred to METAVIR fibrosis stages)(Figure 2, Table S2). Fibrosis grade F0 was considered to be the negative control because these samples were derived from patients with no finding of liver fibrosis. In zebrafish, most highly tissue-specific miRNAs are expressed during embryonic development; approximately 30% of all miRNAs are expressed at a given time point in a given tissue [13]. In mammals, the 20–30% miRNA call rate has recently been validated [14]. Such analysis revealed that the diversity of miRNA expression level among specimens was small. Therefore, we focused on miRNAs with a fold change in mean expression level greater than 1.5 ($p<0.05$) in the two arbitrary groups of liver fibrosis.

Expression of several miRNAs was dramatically different among grades of fibrosis. In the mice study 11 miRNAs were related to the progression of liver fibrosis (mmu-let-7e, miR-125-5p, 199a-5p, 199b, 199b*, 200a, 200b, 31, 34a, 497, and 802). In the human study 10 miRNAs were extracted, and the change in their expression level varied significantly between F0 and F3 (F0<F3: hsa-miR-146b, 199a, 199a*, 200a, 200b, 34a, and 34b, F0>F3: hsa-miR-212, 23b, and 422b). The expression level of 6 miRNAs was significantly different between F0 and F2 (F0<F2: hsa-miR-146b, 200a, 34a, and 34b, F0>F2: hsa-miR-122 and 23b). 5 extracted miRNAs had an expression level that was significantly different between F1 and F2 (F1<F2: hsa-miR-146b, F1>F2: hsa-miR-122, 197, 574, and 768-5p). The expression level of 9 miRNAs changed significantly between F1 and F3 (F1<F3: hsa-miR-146b, 150, 199a, 199a*, 200a, and 200b, F1>F3: hsa-miR-378, 422b, and 768-5p). The miRNAs related to liver fibrosis were extracted using two criteria: similar expression pattern in both the human and the mice specimens and shared sequence between human and mouse. We compared the sequences of mouse miRNAs as described on the Agilent Mouse MiRNA array Version 1.0 (miRbase Version 10.1) and human miRNAs as described on the Agilent Human MiRNA array Version 1.5 (miRbase Version 9.1). The sequences of mmu-miR-199a-5p, mmu-miR-199b, mmu-miR-199b, mmu-miR-200a, and mmu-miR-200b in mouse miRNA corresponded to the sequences of hsa-miR-199a, hsa-miR-199a*, hsa-miR-199a, hsa-miR-200a, and hsa-miR-200b in human miRNA, respectively (Table S3).

Validation of the microarray result by real-time qPCR

The 4 human miRNAs (miR-199a, miR-199a*, miR-200a, and miR-200b) with the largest difference in fold change between the F1 and F3 groups were chosen to validate the microarray results using stem-loop based real-time qPCR. The result of real-time qPCR supported the result of that microarray analysis. The expression level of these 4 miRNAs was significantly different between F0 and F3 and spearman correlation analysis also showed that the expressions of these miRNAs were strongly and positively correlated with fibrosis grade ($n = 105$, $r = 0.498$(miR-199a), 0.607(miR-199a*), 0.639(miR-200a), 0.618(miR-200b), p-values<0.0001) (Figure 3).

Over expression of miR-199a, 199a*, 200a, and 200b was associated with the progression of liver fibrosis

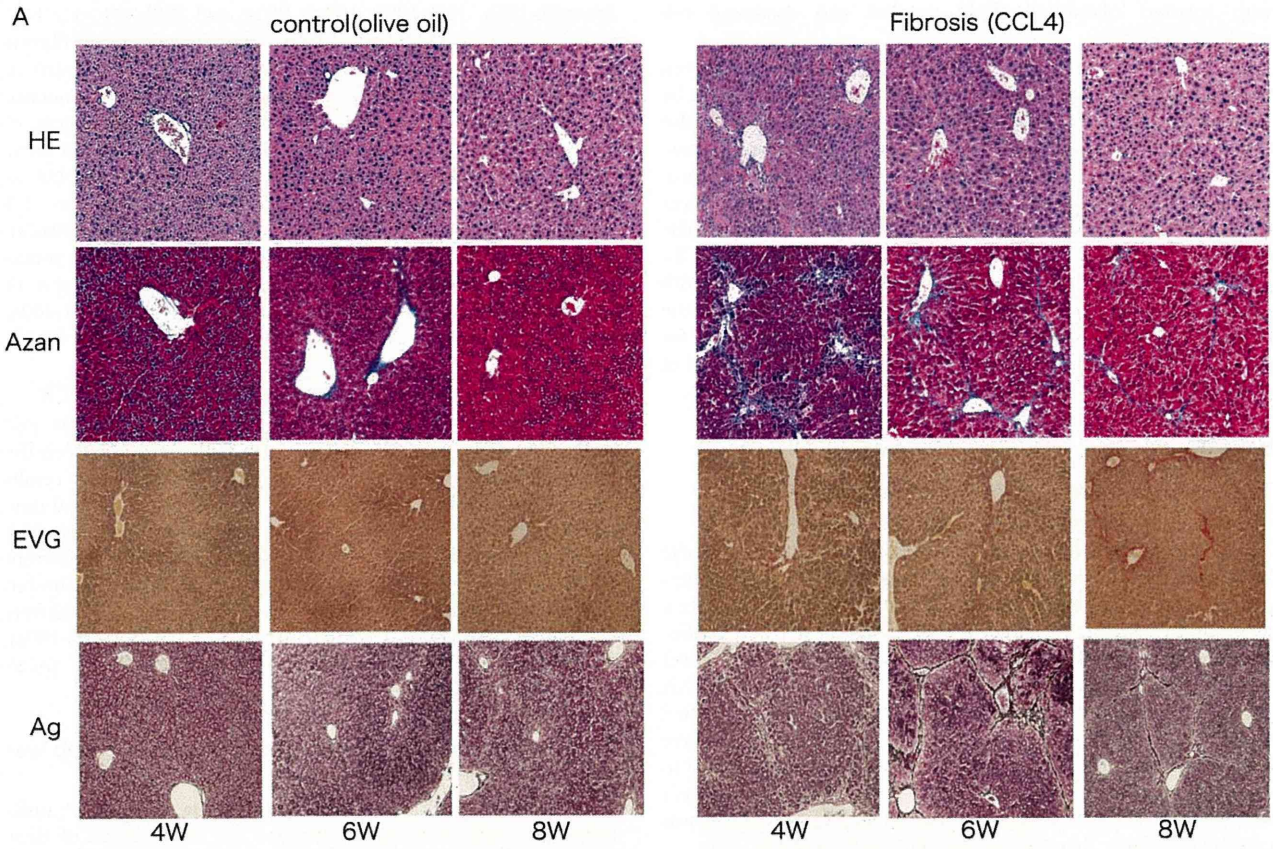
In order to reveal the function of miR-199a, miR-199a*, miR-200a, and miR-200b, we investigated the involvement of these miRNAs in the modulation of fibrosis-related gene in LX-2 cells. The endogenous expression level of these 4 miRNAs in LX2 and normal liver was low according to the microarray study (Figure S2). Transforming growth factor (TGF)β is one of the critical factors for the activation of HSC during chronic inflammation [15] and TGFβ strongly induced expression of three fibrosis-related genes include a matrix degrading complex comprised of α1 procollagen, matrix remodeling complex, comprised of metalloproteinases-13 (MMP-13), tissue inhibitors of metalloproteinases-1 (TIMP-1) in LX-2 cells (Figure 4A). Furthermore, overexpression of miR-199a, miR-199a*, miR-200a and miR-200b in LX-2 cells resulted significant induction of above fibrosis-related genes compared with control miRNA (Figure 4B). Finally we validated the involvement of TGFβ in the modulation of these miRNAs. In LX-2 cells treated with TGFβ, the expression levels of miR-199a and miR-199a* were significantly higher than in untreated cells; the expression levels of miR-200a and miR-200b were significantly lower than in untreated cells. Thus, our in vitro analysis suggested a possible involvement of miR-199a, 199a*, 200a, and 200b in the progression of liver fibrosis.

Discussion

Our comprehensive analysis showed that the aberrant expression of miRNAs was associated with the progression of liver fibrosis. We identified that 4 highly expressed miRNAs (miR-199a, miR-199a*, miR-200a, and miR-200b) that were significantly associated with the progression of liver fibrosis both human and mouse. Coordination of aberrant expression of these miRNAs may contribute to the progression of liver fibrosis.

Prior studies have discussed the expression pattern of miRNA found in liver fibrosis samples between previous and present study. In this report and prior mouse studies and the expression pattern of

A



B

miRNA	Fold change CCL4 mouse/control		
	4week	6week	8week
mmu-let-7e	2.92	2.09	2.64
mmu-miR-125a-5p	4.29	2.35	2.67
mmu-miR-199a-5p	2.71	2.12	2.38
mmu-miR-199b	3.25	2.55	2.49
mmu-miR-199b*	5.13	4.47	3.94
mmu-miR-200a	2.23	1.63	2.02
mmu-miR-200b	2.38	2.06	2.20
mmu-miR-31	3.4	2.35	3.19
mmu-miR-34a	3.89	3.22	3.5
mmu-miR-497	2.25	2.00	2.55
mmu-miR-802	2.02	2.87	2.74

Figure 1. The change of liver fibrosis in mouse model. A. Representative H&E-stained, Azan-stained, Ag-stained, and EVG-stained histological sections of liver from mice receiving olive oil alone or CCL_4 in olive oil. Magnification is ×10. B. The expression level of mmu-miRNA in mouse liver with olive oil or CCL_4 at 4W, 6W, and 8W respectively, by microarray analysis.
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3 miRNAs (miR-199a-5p, 199b*, 125-5p) was found to be similar while the expression pattern of 11 miRNAs (miR-223, 221, 24, 877, 29b, 29a, 29c, 30c, 365, 148a, and 193) was partially consistent with fibrosis grade [16]. In low graded liver fibrosis, the low expression pattern of 3 miRNAs (miR-140, 27a, and 27b) and the high expression pattern of 6 miRNAs in rat miRNAs (miR-29c*, 143, 872, 193, 122, and 146) in rat miRNA was also similar to our mouse study (GEO Series accession number GSE19865) [11] [12] [17].

The results in this study and previously completed human studies reveal that the expression level of miR-195, 222, 200c, 21, and let-7d was higher in high graded fibrotic liver tissue than in low graded fibrotic liver tissue. Additionally, the expression level of miR-301, 194, and 122 was lower in the high graded fibrotic liver tissue than in low graded fibrotic liver tissue [18] [19] [20](GEO Series accession number GSE16922). This difference in miRNA expression pattern may be contributed to (1) the difference of microarray platform, (2) difference of analytic procedure, and (3) the difference of the species (rat, mouse, and human).

The miR-199 and miR-200 families have are circumstantially related to liver fibrosis. TGFβ-induced factor (TGIF) and SMAD

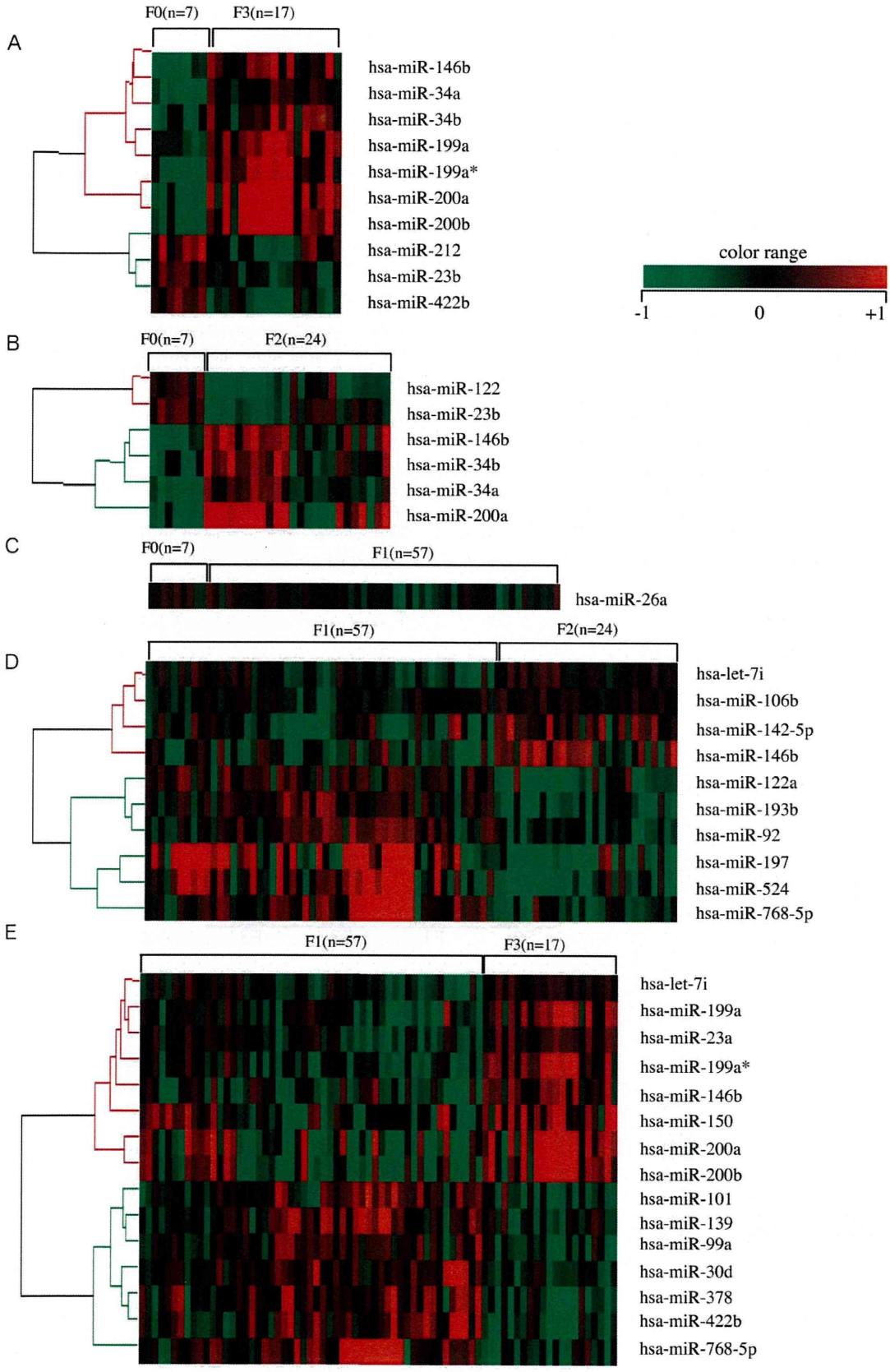
A
F0(n=7)
F3(n=17)
hsa-miR-146b
hsa-miR-34a
hsa-miR-34b
hsa-miR-199a
hsa-miR-199a*
hsa-miR-200a
hsa-miR-200b
hsa-miR-212
hsa-miR-23b
hsa-miR-422b
color range
-1
0
+1
B
F0(n=7)
F2(n=24)
hsa-miR-122
hsa-miR-23b
hsa-miR-146b
hsa-miR-34b
hsa-miR-34a
hsa-miR-200a
C
F0(n=7)
F1(n=57)
hsa-miR-26a
D
F1(n=57)
F2(n=24)
hsa-let-7i
hsa-miR-106b
hsa-miR-142-5p
hsa-miR-146b
hsa-miR-122a
hsa-miR-193b
hsa-miR-92
hsa-miR-197
hsa-miR-524
hsa-miR-768-5p
E
F1(n=57)
F3(n=17)
hsa-let-7i
hsa-miR-199a
hsa-miR-23a
hsa-miR-199a*
hsa-miR-146b
hsa-miR-150
hsa-miR-200a
hsa-miR-200b
hsa-miR-101
hsa-miR-139
hsa-miR-99a
hsa-miR-30d
hsa-miR-378
hsa-miR-422b
hsa-miR-768-5p

Figure 2. Liver fibrosis in human liver biopsy specimen. A. B. C. D. and E. miRNAs whose expression differs significantly between F0 and F3, F0 and F1, F0 and F2, F1 and F2, and F1 and F3, respectively. Relative expression level of each miRNA in human liver biopsy specimen by microarray. Data from microarray were also statistically analyzed using Welch's test and the Bonferroni correction for multiple hypotheses testing. Fold change, p-value are listed in Table S2.
doi:10.1371/journal.pone.0016081.g002

specific E3 ubiquitin protein ligase 2 (SMURF2), both of which play roles in the TGFβ signaling pathway, are candidate targets of miR-199a* and miR-200b, respectively, as determined by the Targetscan algorithm. The expression of miR-199a* was silenced in several proliferating cell lines excluding fibroblasts [21]. Down regulation of miR-199a, miR-199a* and 200a in chronic liver injury tissue was associated with the hepatocarcinogenesis [9]. miR-199a* is also one of the negative regulators of the HCV replication [22]. According to three target search algorithms (Pictar, miRanda, and Targetscan), the miRNAs that may be associated with the liver fibrosis can regulate several fibrosis-related genes (Table S4). Aberrant expression of these miRNAs may be closely related to the progress of the chronic liver disease.

Epithelial-mesenchymal transition (EMT) describes a reversible series of events during which an epithelial cell loses cell-cell contacts and acquires mesenchymal characteristics [23]. Although EMT is not a common event in adults, this process has been implicated in such instances as wound healing and fibrosis. Recent reports showed that the miR-200 family regulated EMT by targeting EMT accelerator ZEB1 and SIP1 [24]. From our observations, overexpression of miR-200a and miR-200b can be connected to the progression of liver fibrosis.

The diagnosis and quantification of fibrosis have traditionally relied on liver biopsy, and this is still true at present. However, there are a number of drawbacks to biopsy, including the invasive nature of the procedure and inter-observer variability. A number of staging systems have been developed to reduce both the inter-observer variability and intra-observer variability, including the METAVIR, the Knodell fibrosis score, and the Scheuer score. However, the reproducibility of hepatic fibrosis and inflammatory activity is not as consistent [25]. In fact, in our study, the degree of fibrosis of the two arbitrary fibrosis groups was classified using the miRNA expression profile with 80% or greater accuracy (data not shown). Thus, miRNA expression can be used for diagnosis of liver fibrosis.

In this study we investigated whether common miRNAs in human and mouse could influence the progression of the liver fibrosis. The signature of miRNAs expression can also serves as a tool for understanding and investigating the mechanism of the onset and progression of liver fibrosis. The miRNA expression profile has the potential to be a novel biomarker of liver fibrosis.

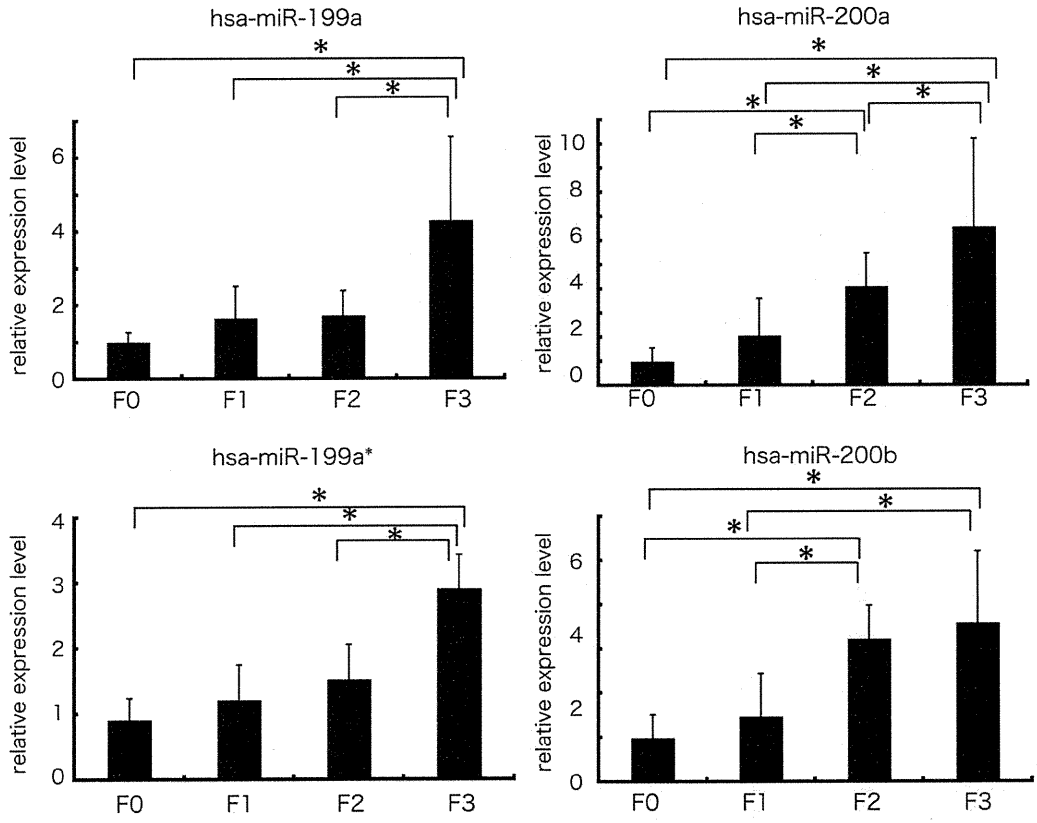

Figure 3. The expression level of miR-199 and 200 families in human liver biopsy specimen by real-time qPCR. Real-time qPCR validation of the 4 miRNAs (miR-199a, miR-199a*, miR-200a, and miR-200b). Each column represents the relative amount of miRNAs normalized to the expression level of U18. The data shown are the means+SD of three independent experiments. Asterisks indicates to a significant difference of $p<0.05$ (two-tailed Student-t test), respectively.
doi:10.1371/journal.pone.0016081.g003

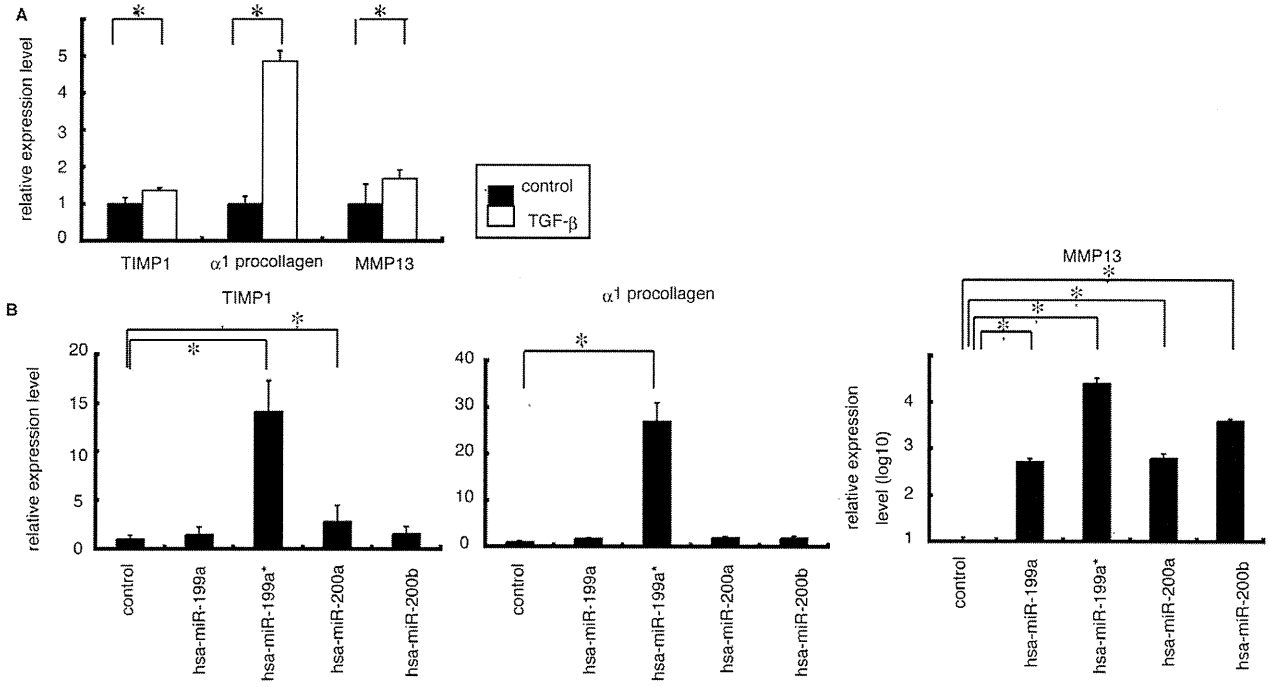

Figure 4. The relationship between expression level of miR-199 and 200 families and expression level of three fibrosis related genes. A. Administration of TGFβ in LX2 cells showed that the expression level of three fibrosis related genes were higher than that in non-treated cells. The data shown are the means+SD of three independent experiments. Asterisk was indicated to the significant difference of $p<0.05$ (two-tailed Student-t test). B. The expression levels of 3 fibrosis related genes in LX2 cells with overexpressing miR-199a, 199a*, 200a, or 200b, respectively were significantly higher than that in cells transfected with control miRNA ($p<0.05$; two-tailed Student t-test).
doi:10.1371/journal.pone.0016081.g004

Moreover miRNA expression profiling has further applications in novel anti-fibrosis therapy in CH.

Materials and Methods

Sample preparation

105 liver tissues samples from chronic hepatitis C patients (genotype 1b) were obtained by fine needle biopsy (Table S1). METAVIR fibrosis stages were F0 in 7 patients, F1 in 57, F2 in 24 and F3 in 17. Patients with autoimmune hepatitis or alcoholic liver injury were excluded. None of the patients were positive for hepatitis B virus associated antigen/ antibody or anti human immunodeficiency virus antibody. No patient received interferon therapy or immunomodulatory therapy prior to the enrollment in this study. We also obtained normal liver tissue from the Liver Transplantation Unit of Kyoto University. All of the patients or their guardians provided written informed consent, and Kyoto University Graduate School and Faculty of Medicine's Ethics Committee approved all aspects of this study in accordance with the Helsinki Declaration.

RNA preparation and miRNA microarray

Total RNA from cell lines or tissue samples was prepared using a *mir*Vana miRNA extraction Kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction. miRNA microarrays were manufactured by Agilent Technologies (Santa Clara, CA, USA) and 100 ng of total RNA was labeled and hybridized using the Human microRNA Microarray Kit protocol for use with Agilent microRNA microarrays Version 1.5 and Mouse microRNA Microarray Kit protocol for use with Agilent microRNA microarrays Version 1.0. Hybridization signals were detected with a DNA microarray scanner G2505B (Agilent Technologies) and the scanned images were analyzed using Agilent feature extraction software (v9.5.3.1). Data were analyzed using GeneSpring GX 7.3.1 software (Agilent Technologies) and normalized as follows: (i) Values below 0.01 were set to 0.01. (ii) In order to compare between one-color expression profile, each measurement was divided by the 75th percentile of all measurements from the same species. The data presented in this manuscript have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE16922 (human) and accession number GSE19865 (mouse).

Real-time qPCR for human miRNA

For detection of the miRNA level by real-time qPCR, TaqMan® microRNA assay (Applied Biosystems) was used to quantify the relative expression level of miR-199a (assay ID. 002304), miR-199a* (assay ID. 000499), miR-200a (assay ID. 000502), miR-200b (assay ID. 002251), and U18 (assay ID. 001204) was used as an internal control. cDNA was synthesized using the Taqman miRNA RT Kit (Applied Biosystems). Total RNA (10 ng/ml) in 5ml of nuclease free water was added to 3 ml of 5× RT primer, 10× 1.5μl of reverse transcriptase buffer, 0.15 μl of 100 mM dNTP, 0.19 μl of RNase inhibitor, 4.16 μl of nuclease free water, and 50U of reverse transcriptase in a total volume of 15 μl. The reaction was performed for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. All reactions were run in triplicate. Chromo 4 detector (BIO-RAD) was used to detect miRNA expression.

Animal and Chronic Mouse Liver Injury Model

Each 5 adult (8-week-old) male C57BL/6J mice were given a biweekly intra-peritoneal dose of a 10% solution of CCL_4 in olive oil (0.02 ml/g/ mouse) for the first 4 weeks and then once a week

for the next 4 weeks. At week 4, 6 or 8, the mice were sacrificed. Partial livers were fixed, embedded in paraffin, and processed for histology. Serial liver sections were stained with hematoxylin-eosin, Azan staining, Silver (Ag) staining, and Elastica van Gieson (EVG) staining, respectively. Total RNA from mice liver tissue was prepared as described previously. All animal procedures concerning the analysis of liver injury were performed in following the guidelines of the Kyoto University Animal Research Committee and were approved by the Ethical Committee of the Faculty of Medicine, Kyoto University.

Cell lines and Cell preparation

The human stellate cell lines LX-2, was provided by Scott L. Friedman. LX-2 cells, which viable in serum free media and have high transfectability, were established from human HSC lines [26]. LX-2 cells were maintained in D-MEM (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum, plated in 60 mm diameter dishes and cultured to 70% confluence. Huh-7 and Hela cells were also maintained in D-MEM with 10% fetal bovine serum. HuS-E/2 immortalized hepatocytes were cultured as described previously [27]. LX-2 cells were then cultured in D-MEM without serum with 0.2% BSA for 48 hours prior to TGFβ1 (Sigma-Aldrich, Suffolk, UK) treatment (2.5 ng/ml for 20 hours). Control cells were cultured in D-MEM without fetal bovine serum.

miRNA transfection

LX-2 cells were plated in 6-well plates the day before transfection and grown to 70% confluence. Cells were transfected with 50 pmol of Silencer® negative control siRNA (Ambion) or double-stranded mature miRNA (Hokkaido System Science, Sapporo, Japan) using lipofectamine RNAiMAX (Invitrogen). Cells were harvested 2 days after transfection.

Real-time qPCR

cDNA was synthesized using the Transcriptor High Fiderity cDNA synthesis Kit (Roche, Basel, Switzerland). Total RNA (2 μg) in 10.4 μl of nuclease free water was added to 1 μl of 50mM random hexamer. The denaturing reaction was performed for 10min at 65°C. The denatured RNA mixture was added to 4 μl of 5× reverse transcriptase buffer, 2 μl of 10 mM dNTP, 0.5 μl of 40U/μl RNase inhibitor, and 1.1 μl of reverse transcriptase (FastStart Universal SYBR Green Master (Roche) in a total volume of 20 μl. The reaction ran for 30 min at 50°C (cDNA synthesis), and five min at 85°C (enzyme denaturation). All reactions were run in triplicate. Chromo 4 detector (BIO-RAD, Hercules, CA, USA) was used to detect mRNA expression. The primer sequences are follows; MMP13 s; 5′-gaggctccgagaaatgcagt-3′, as; 5′-atgccatcgtgaagtctggt-3′, TIMP1 s; 5′-cttggcttctgcactgatgg-3′, as; 5′-acgctggtataaggtggtct-3′, α1-procollagen s; 5′-aacatgaccaaaaaccaaaagtg-3′, as; 5′-catt-gtttcctgtgtcttctgg-3′, and β-actin s; 5′-ccactggcatcgtgatggac-3′, as; 5′-tcattgccaatggtgatgacct-3′. Assays were performed in triplicate, and the expression levels of target genes were normalized to expression of the β-actin gene, as quantified using real-time qPCR as internal controls.

Statistical analyses

Statistical analyses were performed using Student's *t*-test; *p* values less than 0.05 were considered statistically significant. Microarray data were also statistically analyzed using Welch's test and Bonferroni correction for multiple hypotheses testing.

Supporting Information

Figure S1 Time line of the induction of chronic liver fibrosis. Upward arrow indicated administration of olive oil or CCl_4. Downward arrow indicates when mice were sacrificed.
(TIF)

Figure S2 Comparison of the expression level of miR-199 and 200 familes in several cell lines and human liver tissue. Endogenous expression level of miR-199a, 199a*, 200a, and 200b in normal liver and LX2 cell as determined by microarray analysis (Agilent Technologies). Endogenous expression level of same miRNAs in Hela, Huh-7 and, immortalized hepatocyte: HuS-E/2 by previously analyzed data [9].
(TIF)

Table S1 Clinical characteristics of patients by the grade of fibrosis.
(DOCX)

Table S2 Extracted human miRNAs related to liver fibrosis.
(DOCX)

Table S3 Corresponding human and mouse miRNAs.
(DOCX)

Table S4 Hypothetical miRNA target genes according to in silico analysis.
(DOCX)

Author Contributions

Conceived and designed the experiments: YM KS. Performed the experiments: YM HT YH NK. Analyzed the data: MT MK. Contributed reagents/materials/analysis tools: YM HT YH NK. Wrote the paper: YM MT AT FM NK TO.

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Genome wide association studies reveal genetic variants in *CTNND2* for high myopia in Singapore Chinese

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Abstract

Objective—To determine susceptibility genes for high myopia in Singaporean Chinese.

Design—A meta-analysis of two genome wide association (GWA) datasets in Chinese and a follow-up replication cohort in Japanese.

Participants and Controls—Two independent datasets of Singaporean Chinese individuals aged 10–12 years (SCORM -- Singapore Cohort Study of the Risk factors for Myopia: cases=65, controls=238) and aged > 21 years (SP2 -- Singapore Prospective Study Program: cases=222, controls=435) for GWA studies, and a Japanese dataset aged >20 years (cases=959, controls=2128) for replication.

Methods—Genomic DNA samples from SCORM and SP2 were genotyped using various Illumina Beadarray platforms (> HumanHap 500). Single-locus association tests were conducted for each dataset with meta-analysis using pooled z-scores. The top-ranked genetic markers were examined for replication in Japanese dataset. Fisher's P was calculated for the combined analysis of all three cohorts.

Main outcome measures—High myopia, defined by spherical equivalent (SE) ≤ −6.00 diopters (D); controls defined by SE between −0.50D and +1.00D.

Results—Two SNPs (rs12716080 and rs6885224) in the gene *CTNND2* on chromosome 5p15 ranked top in the meta-analysis of our Chinese datasets (meta- $P = 1.14\times10^{-5}$ and meta- $P = 1.51\times10^{-5}$, respectively) with strong supporting evidence in each individual dataset analysis (Max $P = 1.85.x10^{-4}$ in SCORM: Max $P = 8.8\times10^{-3}$ in SP2). Evidence of replication was observed in Japanese dataset for rs6885224 ($P = 0.035$, *meta-P* of three datasets: 7.84×10^{-6}).

Conclusion—This study identified strong association of *CTNND2* for high myopia in Asian datasets. The *CTNND2* gene maps to a known high myopia linkage region on chromosome 5p15.

Keywords

myopia; genome wide association; *CTNND2*; single nucleotide polymorphism; genetics

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Myopia is a common eye disorder and a major public health concern in urban East Asian populations, affecting nearly 40% of Chinese persons aged 40 to 79 years1[−3]. High myopia, defined by spherical equivalent (SE) ≤ −5.00 diopter (D) or SE ≤ −6.00 D for at least one eye, is associated with significant ocular morbidity, including retinal detachment and myopic macular degeneration[4;5].

The genetic etiologic basis of myopia and high myopia is supported by data from familial aggregation, segregation, and twin studies[5–12]. The relative risk of myopia in siblings of a person with myopia (λ_s) has been estimated to be strongest in high myopia (SE ≤ −6.00 D; $\lambda_s = 5 - 20$), and moderate for lower degrees of myopia (SE: −1.00 to −3.00D; λ_s=1.5–3)[5;12]. To date, more than 15 chromosomal regions (or genetic loci, designated as MYP loci) have been mapped for myopia-related phenotypes by genome wide linkage scans, and many candidate genes have been reported by association and sequencing studies[13]. However, no gene implicated in myopia has been consistently replicated.

Genome wide association (GWA) studies have become an important and unbiased approach to aid in the search for causal sequence variants by screening upwards of a million single nucleotide polymorphisms (SNPs) spaced across the genome. This is exemplified by the