AC and AG dinucleotide repeats in the *PAX6* P1 promoter are associated with high myopia

Tsz Kin Ng,[1] Ching Yan Lam,[1] Dennis Shun Chiu Lam,[1] Sylvia Wai Yee Chiang,[1] Pancy Oi Sin Tam,[1] Dan Yi Wang,[1,2] Bao Jian Fan,[1,2] Gary Hin-Fai Yam,[1] Dorothy Shu Ping Fan,[1] Chi Pui Pang[1]

(The first two authors contributed equally to this work.)

[1]*Department of Ophthalmology & Visual Sciences, The Chinese University of Hong Kong, Hong Kong S.A.R.;* [2]*Present affiliation: Department of Ophthalmology, Harvard Medical School, Massachusetts Eye and Ear Infirmary, Boston, MA*

Purpose: The *PAX6* gene, located at the reported myopia locus *MYP7* on chromosome 11p13, was postulated to be associated with myopia development. This study investigated the association of *PAX6* with high myopia in 379 high myopia patients and 349 controls.

Methods: High myopia patients had refractive errors of –6.00 diopters or greater and axial length longer than 26 mm. Control subjects had refractive errors less than –1.00 diopter and axial length shorter than 24 mm. The P1 promoter, all coding sequences, and adjacent splice-site regions of the *PAX6* gene were screened in all study subjects by polymerase chain reaction and direct sequencing. *PAX6* P1 promoter-luciferase constructs with variable AC and AG repeat lengths were prepared and transfected into human ARPE-19 cells prior to assaying for their transcriptional activities.

Results: No sequence alterations in the coding or splicing regions showed an association with high myopia. Two dinucleotide repeats, $(AC)_m$ and $(AG)_n$, in the P1 promoter region were found to be highly polymorphic and significantly associated with high myopia. Higher repeat numbers were observed in high myopia patients for both $(AC)_m$ (empirical $p = 0.013$) and $(AG)_n$ (empirical $p = 0.012$) dinucleotide polymorphisms, with a 1.327-fold increased risk associated with the $(AG)_n$ repeat (empirical $p = 0.016$; 95% confidence interval: 1.059–1.663). Luciferase-reporter analysis showed elevated transcription activity with increasing individual $(AC)_m$ and $(AG)_n$ and combined $(AC)_m(AG)_n$ repeat lengths.

Conclusions: Our results revealed an association between high myopia and AC and AG dinucleotide repeat lengths in the *PAX6* P1 promoter, indicating the involvement of *PAX6* in the pathogenesis of high myopia.

Myopia, one of the most common refractive errors of the eye worldwide, is an important public health issue, especially in Asia, because of its higher prevalence in Asians than in other populations [1]. The progression of myopia in Chinese children in Hong Kong and Singapore is also much higher than in Caucasians [2,3]. In Hong Kong, the prevalence of myopia in Chinese schoolchildren aged 11–16 was 36.7%, according to a 2004 report, which is several times higher than among Caucasian children of similar ages [4]. The prevalence of high myopia, defined as a refractive error equal to or greater than –6.00 diopters (D), is also higher in Chinese than in Caucasians [5,6]. Individuals with high myopia are more prone to develop serious ocular complications, such as retinal detachment, glaucoma, premature cataracts, and macular degeneration, which may lead to visual impairment or even blindness [7-10].

Myopia is a complex disorder. Multiple interacting environmental and genetic causes are implicated. Myopia development in schoolchildren has been attributed to environmental factors, such as near work, reading habits, and school achievement [3,11,12]. In addition, high heritability of refractive errors has been observed in dizygotic and monozygotic twin studies [13-17]. Family and sibling studies have shown that children of myopic parents have greater chances of developing myopia than those with nonmyopic parents [11,18]. Twenty-four chromosomal loci have been identified for myopia: Xq28 (*MYP1)* [19], 18p11.31 (*MYP2)* [20,21], 12q21-31 (*MYP3)* [22], 7q36 (*MYP4)* [23], 17q21-22 (*MYP5)* [24], 22q37.1 (*MYP6)* [25], 11p13 (*MYP7)* [26], 3q26 (*MYP8)* [26], 4q12 (*MYP9)* [26], 8p23 (*MYP10)* [26], 4q22-q27 (*MYP11)* [27], 2q37.1 (*MYP12)* [28], Xq23 (*MYP13)* [29], 1p36 (*MYP14)* [30], 10q21.2 *(MYP15)* [31], 5p15.33-p15.2 (*MYP16)* [32], 7p15 (*MYP17)* [33,34], 14q22.1-q24.2 (*MYP18*) [35], 15q12-13 [36], 21q22.3 [37], 12q24 [38], 4q21 [38], 9q34.11 [39] and 2q37 [40]. Among them, *MYP1–5,11–13,16*, and *18* are linked to high myopia, and *MYP2,11,13, 16*, and *18* are found in the Chinese population. Some candidate genes have been postulated for myopia, such as *TGIF* [41], *HGF* [42], *MMP3* [43], *MMP9* [43], *COL1A1* [44], *COL2A1* [45], *TGFB1* [46], *TGFB2* [47], *LUM* [48], and *CMET* [49].

Correspondence to: Prof. C.P. Pang, Department of Ophthalmology & Visual Sciences, The Chinese University of Hong Kong, University Eye Center, Hong Kong Eye Hospital, 147K Argyle Street, Kowloon, Hong Kong; Phone: +852 27623129; FAX: +852 27159490; email: cppang@cuhk.edu.hk

A genome-wide scan in dizygotic twins revealed a susceptibility locus for myopia on chromosome 11p13 [26]. The *PAX6* gene at this locus, a member of the paired-domain PAX family, has been postulated as a candidate gene for myopia. *PAX6* is expressed in the human eye [50] and plays an evolutionarily conserved role in ocular development [51-53]. *PAX6* mutations are associated with ocular disorders, such as aniridia (OMIM 106210), cataracts (OMIM 604219), Peters anomaly (OMIM 604229), and optic nerve hypoplasia (OMIM 16550). *PAX6* encodes a transcriptional regulator containing the DNA-binding paired domain, paired-type homeodomain, and COOH-terminal transactivation domain. The Pax6 protein regulates cell adhesion molecules, cell-to-cell signaling molecules, hormones, and structural proteins [54] through interactions with transcription factors such as Mitf [55] and Sox2 [56]. Transcription of *PAX6* is regulated by at least two promoters, P0 and P1 [57-60]. Within the P1 promoter (promoter B in Okladnova et al. [59]), two dinucleotide repeats, $(AC)_m$ and $(AG)_n$, are located about 1 kb from the transcription start site [58] and are highly polymorphic in Caucasians. The poly AC and poly AG repeats are independently polymorphic [60]. Luciferase analysis in Cos-7 cells has shown that the longer the combined length of the AC and AG repeats, the higher the transcriptional activity, implying that the length of this dinucleotide repeat might influence the transcriptional activity of promoter B, or P1, and subsequently the transcription of *PAX6*.

Pax6 levels are tightly controlled. Both overexpression and haploinsufficiency lead to abnormal phenotypes [61-63]. Polymorphisms or mutations in the *PAX6* promoter could influence *PAX6* expressions that ultimately lead to a disease phenotype. However, although *PAX6* has been postulated to be a candidate gene for myopia, several studies in Caucasian populations could not find an association between *PAX6* and myopia [26,45,64]. Still, an Australian study suggested *PAX6* mutations might be associated with high myopia [65]. Intronic sequence alterations (SNPs) in *PAX6* have been reported to associate with high myopia in Han Chinese nuclear families [66] and with extreme myopia in a Taiwan Chinese population [67], but not in Caucasians. To attest the association between *PAX6* and high myopia, we should look for mutations that may affect *PAX6* expressions. We therefore screened for sequence alterations in the P1 promoter, coding exons, and adjacent splice-site regions of *PAX6* in unrelated high myopia patients and control subjects. We also examined transcriptional effects of dinucleotide repeats within the P1 promoter in cultured human APRE-19 cells by a luciferase-reporter assay and predicted the presence of transcription factor binding sites within the repeats.

METHODS

Study subjects: We recruited 379 unrelated Han Chinese patients with high myopia at the Hong Kong Eye Hospital. They were given complete ophthalmoscopic examinations. None of them had known diseases predisposing them to myopia, such as Stickler or Marfan syndromes. Their refractive errors were equal to or greater than –6.00 D, and their axial length was longer than 26 mm. We also recruited 349 unrelated Chinese control subjects who visited the hospital for ophthalmic examinations. They had no eye diseases except senile cataracts and slight floaters. All of them had refractive errors of less than –1.00 D and axial length shorter than 24 mm. The study protocol was approved by the Ethics Committee for Human Research at the Chinese University of Hong Kong and was in accordance with the tenets of the Declaration of Helsinki. Informed consent was obtained from the study subjects after explanation of the nature and possible consequences of the study.

PAX6 genotyping: The whole blood specimens (5 ml) from all the patients and controls were collected in EDTA tube and stored at -80 °C for fewer than two months. Genomic DNA was extracted (QIAamp DNA kit; Qiagen, Hiden, Germeny) according to the supplier's instructions. All samples were screened for sequence alterations in the P1 promoter region flanking –3,433 to –118, coding exons, and intron-exon boundaries of *PAX6* (ENSG00000007372 and ENST00000241001; Ensembl genome browser) by polymerase chain reaction (PCR) with primer sets [61]. PCR was performed in a final volume of 25 μl containing 1X PCR buffer (Invitrogen™ Life Technology, Carlsbad, CA), 1.5 mM $MgCl_2$, 0.2 mM of dNTP (Roche, Indianapolis, IN), 0.2 mM of each primers, 0.5 U of Platinum® Taq DNA polymerase (Invitrogen). After the initial denaturation at 95 °C for 2 min, 40 PCR cycles were conducted: 95 °C for 45 s, 57 °C for 45 s and 72 °C for 45 s. The final extension lasted for 5 min at 72 °C. Direct sequencing was performed using a BigDye Terminator Cycle Sequencing Reaction Kit (v3.1, Applied Biosystems, Foster City, CA) on an ABI 3130XL capillary DNA sequencer (Applied Biosystems).

Construction of PAX6 P1 promoter-luciferase constructs: A 1,851 bp genomic fragment (from –1278 to +573) containing the *PAX6* P1 promoter was cloned into an empty pGL3-Basic vector, pGL3 (Promega, Madison, WI) between the SacI and BglII sites (OriGene Technologies, Rockville, MD). Constructs with different repeat lengths were generated. Genomic DNA from the study subjects was amplified by PCR (forward primer 5'-ACA CAC AGA TGA CCG GTG G-3'; reverse primer 5'-AAG CCT AGG CCG AGA GGA-3'). AgeI and AvrII digested products were ligated into a linearized pGL3-Basic vector containing the P1 promoter (pGL3-Pax6p). A positive control construct was made by cloning a pCMV5 promoter [68] into the pGL3-Basic vector (pGL3-pCMV). All constructs were verified by direct sequencing.

Cell culture and transfection: The human retinal pigment epithelial cell line ARPE-19 (American Type Culture Collection, Manassas, VA) [69] was cultured in Dulbecco's modified Eagle's medium and F-12 nutrient mixture

supplemented with 10% fetal bovine serum (Gibco BRL, Rockville, MD). Cells were plated in 60 mm tissue culture dishes at a density of $2–3\times10^5$ cells/dish one day before transfection. At 60–80% confluence, cells were transfected with 2 μg luciferase constructs in 6 μl FuGene HD (Roche) transfection reagent per dish. Empty pGL3 and pGL3-pCMV were used as negative and positive controls, respectively. At 36 h after transfection, cell lysates were extracted using Cell Culture Lysis Reagent (Promega, Madison, WI) for immunoblotting.

Immunoblotting: The denatured cell lysates of the transfected cells were resolved on 10% SDS-polyacrylamide gel and electro-transferred to nitrocellulose membranes for probing with a rabbit polyclonal primary antibody against firefly luciferase (Sigma-Aldrich, St. Louis, MO) and a secondary antibody against rabbit IgG conjugated with horseradish peroxidase (Jackson Immuno Res., West Grove, PA). The chemiluminescence was detected by an enhanced chemiluminescence system (Amersham Pharmacia, Cleveland, OH) and quantified by ChemiDoc (BioRad, Hercules, CA). Normalized luciferase intensities were calculated by dividing the quantified luciferase intensities by the housekeeping β-actin intensities. Triplicates were performed.

Statistical analysis: The χ^2 test or Fisher exact test was used to compare the allele and genotype frequencies of SNPs in patients and control subjects. For the comparison of $(AC)_m$ and $(AG)_n$ repeat alleles and genotypes between high myopia patients and control subjects, the χ^2 test was performed using the CLUMP program (version 2.3) [70]. For multiple testing corrections, 10,000 Monte Carlo permutations were chosen to simulate the empirical significance levels of the statistics produced by the program, resulting in an empirical p-value. Due to low frequencies of some alleles, and in order to determine whether the transcriptional activities were affected by the thresholds, $(AC)_m$ and $(AG)_n$ repeats were collapsed into groups for association study and immunoblotting analysis [71,72]. The risk of high myopia was also determined by odds ratio using the χ^2 test. Activity of each allelic construct was expressed relative to $(AC)_{20}(AG)_6$. One-way ANOVA and independent T-testing were used to compare the means among $(AC)_m$ groups and between $(AG)_n$ repeats, respectively. SNP-trait association, odds ratio calculation, and immunoblotting analysis were performed on SPSS version 16.0 (SPSS Science, Chicago, IL). Significance was defined as p < 0.05.

Transcription factor binding site prediction: The DNA sequence of the cloned *PAX6* P1 promoter was used to predict transcription factor binding sites. The Transcription Element Search System (TESS: University of Pennsylvania, Philadelphia, PA) [73,74] was used to predict the transcription factors that would bind to the region of the dinucleotide repeats in the *PAX6* P1 promoter. Predictions for different lengths of dinucleotide repeats were also performed. As in the statistical analysis for immunoblotting, $(AC)_{20}(AG)_6$ was set

RESULTS

In our study cohort, high myopia patients had a mean age of 39.52±14.96 years and a male-to-female ratio of 1.2:1. Refractive errors ranged from –6.00 to –30.00 D. For the controls, the mean age was 64.85±14.85 years, with a male-to-female ratio of 1.6:1. There was no significant difference in the sex ratio between high myopia patients and controls.

Two sequence changes were identified in coding exons with the intron-exon boundary of *PAX6*. One novel heterozygous silent variant, 678A>G (R67R), was found in one high myopia patient, and a noncoding sequence change, rs667773, was found in both patients and controls. Allelic and genotypic frequencies of both polymorphisms showed no significant difference (p > 0.05) between patients and controls (data not shown). Within the P1 promoter region, 20 polymorphisms were identified, with no significant difference in frequencies between patients and controls: –186C>T, –215G>A, –242G>A, –263A>G, –292A>G, –331A>G, –337A>T, –354A>G, –382G>A, –407G>A, –409G>A, –692A>G, –758C>T, –782A>G, –933C>G, –3050C>A, –3070C>A, –3078A>G, –3090C>T, and –3282T>C (data not shown). For -186C>T, -292A>G, -331A>G, -933C>G, and -3282T>C, each SNP was only found in 1 high myopia patient. Therefore, they were statistically not significant under Pearson's χ2 test (p > 0.05).

Within the *PAX6* P1 promoter, two dinucleotide repeats, $(AC)_m$ and $(AG)_n$, were observed about 1 kb from the transcription start site, both highly polymorphic (Table 1). The AC repeats ranged from 16 to 26 in high myopia patients and from 7 to 26 in control subjects, while 5 to 8 AG repeats were observed in patients and 4 to 8 in controls. The median numbers of AC and AG repeats were 20 and 6, respectively, in both patients and controls. Distribution of the allele frequencies was slightly skewed in patients for both AC and AG repeats. Allele frequencies of the AC and AG repeats were significantly different between patients and controls (empirical p = 0.013 and 0.012, respectively; Table 1). Because the frequencies of some of the alleles were low, the AC and AG repeats were collapsed into groups. The grouped repeat lengths were longer in patients than in controls (empirical p = 0.016 for $(AC)_m$ and empirical *p* = 0.016 for $(AG)_n$; Table 2). In terms of risk analysis, individuals with $(AG)_{7-8}$ repeats had a 1.327-fold increased risk of developing high myopia compared with the those with $(AG)_{4-6}$ repeats (empirical p = 0.016; 95% confidence interval = 1.059–1.663). Both grouped AC and grouped AG genotypes were significantly different between high myopia patients and control (empirical p = 0.004 and 0.039, respectively; Table 3).

We found that the dinucleotide repeats affected the transcriptional activity of the *PAX6* P1 promoter (Figure 1). For a given $(AG)_n$ repeat length, elevated transcriptional

TABLE 1. ALLELIC FREQUENCIES OF *PAX6* P1 PROMOTER DINUCLEOTIDE REPEATS IN HIGH MYOPIA (HM) AND CONTROL SUBJECTS.

$(AC)_m$ repeat	Allelic count (%)		Empirical p-value	$(AG)_n$ repeat	Allelic count (%)		Empirical p-value
	HM n=750	Control n=678			HM n=758	Control n=698	
$(AC)_7$	0 (0.0)	1 (0.1)	0.013	$(AG)_4$	0 (0.0)	1 (0.1)	0.012
$(AC)_{15}$	0 (0.0)	2 (0.3)		$(AG)_5$	45 (5.9)	51 (7.3)	
$(AC)_{16}$	10 (1.3)	9 (1.3)		$(AG)_6$	464 (61.2)	458 (65.6)	
$(AC)_{17}$	43 (5.7)	41 (6.0)		$(AG)_7$	218 (28.8)	176 (25.2)	
$(AC)_{18}$	80 (10.7)	67 (9.9)		$(AG)_8$	31 (4.1)	12 (1.7)	
$(AC)_{19}$	100 (13.3)	138 (20.4)					
$(AC)_{20}$	155 (20.7)	134 (19.8)					
$(AC)_{21}$	149 (19.9)	99 (14.6)					
$(AC)_{22}$	161 (21.5)	138 (20.4)					
$(AC)_{23}$	29 (3.9)	33 (4.9)					
$(AC)_{24}$	13 (1.7)	13 (1.9)					
$(AC)_{25}$	6 (0.8)	2 (0.3)					
$(AC)_{26}$	4 (0.5)	1 (0.1)					

TABLE 2. ALLELIC FREQUENCIES OF *PAX6* P1 PROMOTER GROUPED DINUCLEOTIDE REPEATS, $(AC)_m$ AND $(AG)_n$, IN HIGH MYOPIA (HM) AND CONTROL SUBJECTS.

Grouped $(AC)_m$ repeat	Allelic count (%)		Empirical p-value	Grouped $(AG)_n$ repeat	Allelic count (%)		Empirical p-value
	HM n=750	Control n=678			HM n=758	Control n=698	
$(AC)_{Below\ 20-22}$	233 (31.1)	258 (38.1)	0.016	$(AG)_{4-6}$	509 (67.2)	510 (73.1)	0.016
$(AC)_{20-22}$	465 (62.0)	371 (54.7)		$(AG)_{7-8}$	249 (32.8)	188 (26.9)	
$(AC)_{Above\ 20-22}$	52 (6.9)	49 (7.2)					

TABLE 3. GENOTYPIC FREQUENCIES OF *PAX6* P1 PROMOTER GROUPED DINUCLEOTIDE REPEATS IN HIGH MYOPIA (HM) AND CONTROL SUBJECTS.

Grouped $(AC)_m$ genotype	Genotypic count (%)		Empirical p-value	Grouped $(AG)_n$ genotype	Genotypic count (%)		Empirical p-value
	HM n=375	Control n=339			HM n=379	Control n=349	
$(AC)_{Below\ 20-22}$ / $(AC)_{Below\ 20-22}$	16 (4.3%)	40 (11.8%)	0.004	$(AG)_{4-6}$ / $(AG)_{4-6}$	173 (45.6%)	192 (55.0%)	0.039
$(AC)_{Below\ 20-22}$ / $(AC)_{20-22}$	178 (47.5%)	149 (44.0%)		$(AG)_{4-6}$ / $(AG)_{7-8}$	163 (43.0%)	126 (36.1%)	
$(AC)_{Below\ 20-22}$ / $(AC)_{Above\ 20-22}$	24 (6.4%)	29 (8.6%)		$(AG)_{7-8}$ / $(AG)_{7-8}$	43 (11.3%)	31 (8.9%)	
$(AC)_{20-22}$ / $(AC)_{20-22}$	130 (34.7%)	103 (34.7%)					
$(AC)_{20-22}$ / $(AC)_{Above\ 20-22}$	26 (6.9%)	16 (4.7%)					
$(AC)_{Above\ 20-22}$ / $(AC)_{Above\ 20-22}$	1 (0.3%)	2 (0.6%)					

activity was observed with increasing length of $(AC)_m$ repeats ($p = 0.004$, one-way ANOVA; post-hoc tests adjusted by Tukey HSD: $(AC)_{Below20–22}$ versus $(AC)_{20–22}$, $p = 0.033$; and $(AC)_{Below20–22}$ versus $(AC)_{Above20–22}$, $p = 0.004$; Figure 1A,B). Similarly, at a given $(AC)_m$ repeat length, transcriptional activity of $(AG)_8$ was increased when compared with $(AG)_6$, although the increase was not significant, likely due to the substantial standard deviation ($p = 0.205$, independent T-test; Figure 1C,D). For combined repeats of the same length, transcriptional activity of $(AC)_{23}(AG)_6$ was similar to that of $(AC)_{21}(AG)_8$ ($p = 0.627$, independent T-test; Figure 1E,F). Thus, both AC and AG repeats contributed to the transcriptional activity of the *PAX6* P1 promoter.

Our luciferase-reporter analysis showed that transcription activity increased with AC and AG repeat length. This phenomenon may be due to influences of transcription factor binding sites within this region. Thus, we used $(AC)_{20}(AG)_6$ as a reference and predicted one binding site for T-cell factor/Lymphoid enhancer factor family transcription factors, one glucocorticoid receptor binding site, and four transcription factor (TF) II-I binding sites (Figure 2B). With decreasing AG repeat lengths, the T-cell factor/Lymphoid enhancer factor and glucocorticoid receptor sites were unchanged, but the TFII-I sites were reduced. Only two predicted TFII-I sites were observed in $(AC)_{15}(AG)_4$ (Figure 2A). No alteration was observed with a decrease in AC repeat
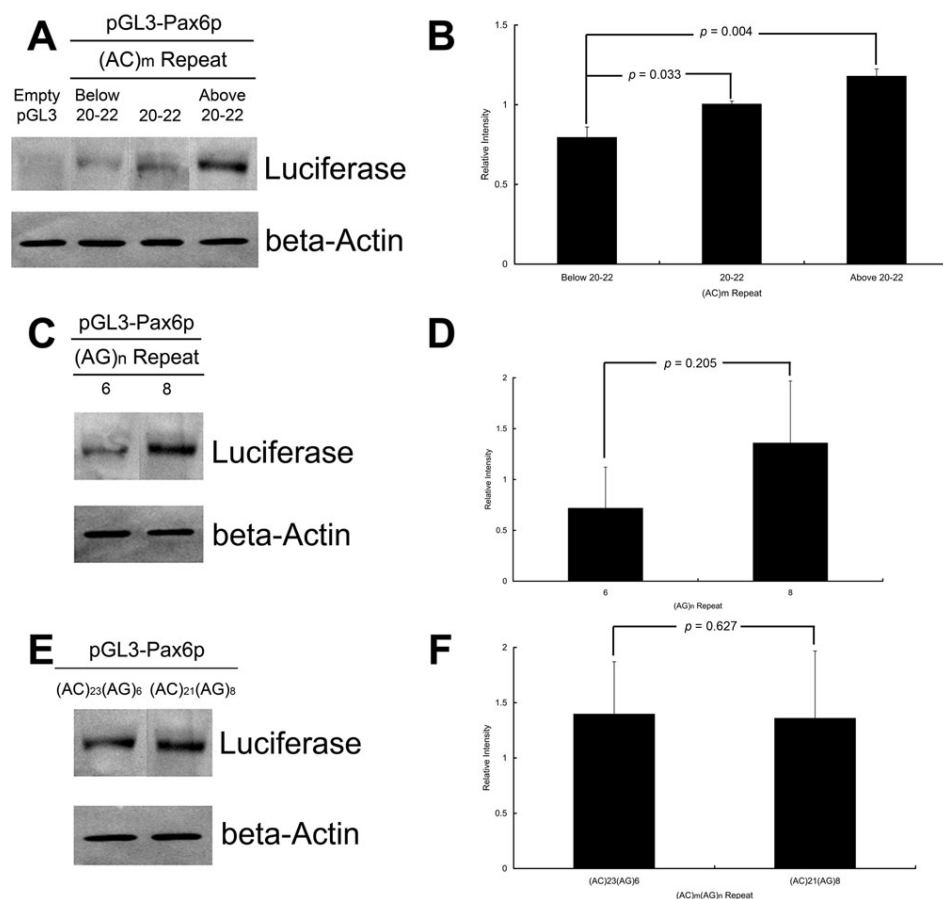
Figure 1. Transcriptional activity of dinucleotide repeats in the *PAX6* P1 promoter. A 1,851 bp genomic fragment (from −1278 to +573) containing the *PAX6* P1 promoter with different dinucleotide repeats was cloned into an empty pGL3-Basic vector (pGL3) and transfected into ARPE-19 cells. The activity of each allelic construct is expressed relative to the construct $(AC)_{20}(AG)_6$. Data are represented as mean±SD for five independent experiments. **A** and **B:** Immunoblotting results and a bar chart show relative luciferase activity for grouped $(AC)_m$ repeats with a stable $(AG)_6$. **C** and **D:** Immunoblotting results and a bar chart show relative luciferase activity for $(AG)_n$ repeats with $(AC)_{21}$. **E** and **F:** Immunoblotting results and a bar chart show relative luciferase activity for combined $(AC)_m(AG)_n$ repeats.
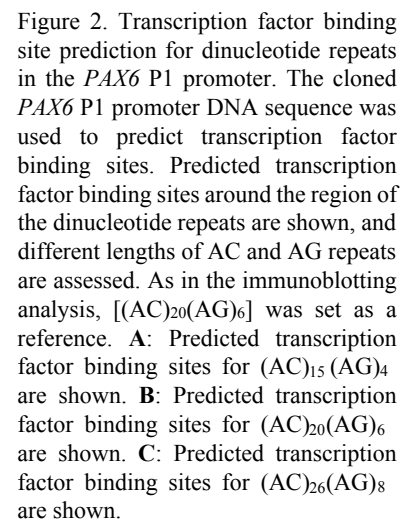
lengths. Accordingly, more TFII-I sites were predicted with increasing AG repeat lengths. Multiple sites for Wilms' tumor transcription factor without lysine-threonine-serine [Wt1(−KTS)] were observed with an increase in AC repeat length, and one GAGA factor binding site appeared with an increase in AG repeat length. In $(AC)_{26}(AG)_8$, six TFII-I sites, six Wt1(−KTS) sites, and one GAGA factor site were predicted (Figure 2C).

DISCUSSION
We found no myopia mutations in the coding regions and splice sites in *PAX6* in our cohort of Chinese high myopia patients. Some SNPs were detected in the P1 promoter, exon 7, and intron 10, but these were not statistically significant (data not shown). In a recent report, two intronic SNPs (rs3026390 and rs3026393, located in introns 12 and 13, respectively) have been shown to be associated with high myopia in Han Chinese nuclear families [66]. SNP rs667773, located in intron 10, is in the same linkage disequilibrium block with rs3026390 and rs3026393 [66]. However, in our study, no significant association was found for rs667773 between high myopia patients and controls, which was consistent with a previous case-control association study in a Taiwan Chinese population [67]. The discrepancy

might be due to the much lower minor allele frequency of rs667773 (0.137) than of rs3026390 and rs3026393 (0.472 and 0.493, respectively) [66]. Other studies have suggested that rs667773, as a neural polymorphism, is an unlikely cause of overt phenotypes such as aniridia [75,76].

The $(AC)_m(AG)_n$ dinucleotide repeat sequence, located about 1 kb from the transcription start site of the *PAX6* P1 promoter, is highly polymorphic. The AC dinucleotide polymorphism ranged from 18 to 31 repeats and AG ranged from 5 to 7 repeats in a Caucasian population [60]. In our Chinese cohort, the AC repeats ranged from 7 to 26 and the AG repeats from 4 to 8 (Table 1). The allele size of the AG repeats was similar in Caucasians and Chinese, but the AC repeat length was longer in Caucasians. Notably, one $(AC)_7$ allele was found in a control subject, far from the common range of repeats between 15 and 26. In addition, many of the dinucleotide repeats were heterozygous in both poly AC and poly AG repeats (AC: 55.3% in controls and 75.9% in patients; AG: 42.9% in controls and 53.4% in patients). The observed heterozygosity rate was 65% in a Caucasian population [60]. Although the allele number in that study was defined as combined units of AC and AG repeats instead of independent AC and AG alleles, the trend of heterozygosity was similar to that in our work. These two dinucleotide repeats

A

```
ATACCGACAT CGCCAGTGGG ACACACACAC ACACACACAC ACACACACAC
           ====(8.00) NF-1                         ===···LEF-1,TCF-1(P),TCF-1,TCF-1A,TCF-1B,TCF-1C,TCF-1E,TCF-1F,TCF-1G,TCF-2alpha
           === ===(12.00) GR                       =···GR

AGAGAGAGAA TCCCTCCCAG CATTGGTCAT CCGCCCCCCC ACCCAGGCTT
···==(8.00) LEF-1,TCF-1(P),TCF-1,TCF-1A,TCF-1B,TCF-1C,TCF-1E,TCF-1F,TCF-1G,TCF-2alpha
···====(10.00) GR
    ======(9.9296) TFII-I
     ======(9.9296) TFII-I
              ======(14.00) MAZ
           =====(10.00) CTCF
```

B

```
ATACCGACAT CGCCAGTGGG ACACACACAC ACACACACAC ACACACACAC
           ====(8.00) NF-1
           === ===(12.00) GR

ACACACACAC AGAGAGAGAG AGAATCCCTC CCAGCATTGG TCATCCGCCC
      === ==(8.00) LEF-1,TCF-1(P),TCF-1,TCF-1A,TCF-1B,TCF-1C,TCF-1E,TCF-1F,TCF-1G,TCF-2alpha
      = ====(10.00) GR
       ======(9.9296) TFII-I
          ======(9.9296) TFII-I
            ======(9.9296) TFII-I
           ==== ==(9.9296) TFII-I
                 =====(10.00) CTCF
                 ===== ==(14.00) MAZ
```

C

```
ATACCGACAT CGCCAGTGGG ACACACACAC ACACACACAC ACACACACAC
           ====(8.00) NF-1
           === ===(12.00) GR
             ========= =========== ===========···WT1_-KTS
             ========= =========== ===========···WT1_-KTS
             ======    =========== ===========···WT1_-KTS
               === =========== ===========···WT1_-KTS
               = =========== ===========···WT1_-KTS
                 =========== ===========···WT1_-KTS

ACACACACAC ACACACACAC ACAGAGAGAG AGAGAGAGAA TCCCTCCCAG
···========= ==(63.3829) WT1_-KTS
···========= ====(63.3829) WT1_-KTS
···========= ======(63.3829) WT1_-KTS
···========= ========(63.3829) WT1_-KTS
···========= =========(63.3829) WT1_-KTS
···========= ==(63.3829) WT1_-KTS
           = ====(8.00) LEF-1,TCF-1(P),TCF-1,TCF-1A,TCF-1B,TCF-1C,TCF-1E,TCF-1F,TCF-1G,TCF-2alpha
             ====(10.00) GR
             ======(9.9296) TFII-I
             ======= ======(26.00) GAGA factor
             ======(9.9296) TFII-I
              ==== ==(9.9296) TFII-I
                == ====(9.9296) TFII-I
                 ======(9.9296) TFII-I
                  ======(9.9296) TFII-I
                       =====(10.00) CTCF
                       ======(14.00) MAZ
```

Figure 2. Transcription factor binding site prediction for dinucleotide repeats in the *PAX6* P1 promoter. The cloned *PAX6* P1 promoter DNA sequence was used to predict transcription factor binding sites. Predicted transcription factor binding sites around the region of the dinucleotide repeats are shown, and different lengths of AC and AG repeats are assessed. As in the immunoblotting analysis, $[(AC)_{20}(AG)_6]$ was set as a reference. **A**: Predicted transcription factor binding sites for $(AC)_{15}(AG)_4$ are shown. **B**: Predicted transcription factor binding sites for $(AC)_{20}(AG)_6$ are shown. **C**: Predicted transcription factor binding sites for $(AC)_{26}(AG)_8$ are shown.

are, therefore, highly polymorphic both in Caucasians and in Chinese.

The *PAX6* P1, containing CCAAT boxes and a TATA-like box, is likely a real promoter [58-60]. We evaluated the influence of $(AC)_m(AG)_n$ dinucleotide repeats on *PAX6* P1 promoter activity by a luciferase-reporter assay and examined the effects of repeat lengths as obtained from our high myopia patients and controls. Since retinal pigment epithelium (RPE) has been shown to have *PAX6* P1 promoter activity [57], we used an RPE cell line, ARPE-19, for transfection. Immunoblotting showed that longer lengths of $(AC)_m$ have a significant trend of increasing luciferase expression compared with shorter lengths (Figure 1A,B), although this was not observed for $(AG)_n$, likely due to the substantial standard deviation (Figure 1C,D).

We confirmed that transcriptional activity of $(AC)_{23}(AG)_6$ was similar to that of $(AC)_{21}(AG)_8$ (Figure 1E,F), suggesting that both $(AC)_m$ and $(AG)_n$ dinucleotide repeats within the *PAX6* P1 promoter contribute to transcriptional activity and might work cooperatively as an unit. Previous studies on luciferase-reporter assays assessed the promoter activity invisibly by a luminometer [60,77]. In our study, we monitored the luciferase-reporter assay by immunoblotting using a commercially available antibody against firefly luciferase and luciferase overexpression by pGL3-pCMV as a positive control. There are technical advantages to this method. The promoter activity could be visualized, and co-transfection with another normalizing vector was not required, as the luciferase intensity could be directly normalized with the housekeeping protein, assuming the same transfection efficiency among the constructs. The limitation of the luciferase-reporter assay is that the effect of the dinucleotide repeats on the transcriptional activity was performed using RPE cells from normal controls, which might not truly reflect the situation in high myopia unless the experiment were performed using cells from a highly myopic individual.

Since levels of Pax6 are tightly controlled, small and seemingly insignificant changes in the levels of Pax6 may lead to significant phenotypic consequences [78]. Moreover, the Pax6 protein could upregulate *PAX6* P1 promoter activity

[77]. Results of our genotyping and promoter activity analyses indicate that longer lengths of dinucleotide repeats increase the expression of *PAX6*, which increases the risk of high myopia. This postulation may be supported by several assertions: (1) *PAX6* gene expression has been shown to be significantly higher in the retinas of optical defocused eyes than in contralateral eyes in the rhesus monkey [79], and expression of *PAX6* was also increased in posthatch chicken eyes with form-deprivation myopia [78]. (2) In another study, the number of dividing retinal progenitor cells, of which *PAX6* is a marker, was highly correlated with axial elongation of the eye, resulting in myopic refractive errors in primates with form-deprivation myopia [80]. (3) Pax6 has been shown to transactivate insulin promoters [81] and promote proinsulin processing [82]. As insulin is a strong stimulator of axial myopia in chicks [83], elevated *PAX6* expression may increase the risk of developing myopia through increased expression of insulin. Chronic hyperinsulinemia has been proposed as a key player in the pathogenesis of juvenile-onset myopia [84]. Although Pax6 also transactivates the glucagon promoter [81], which is a "stop" for myopia [85], insulin might overcome the effects of glucagon in the development of myopia [86].

The transcription factor binding site prediction (Figure 2) showed that an increase in AC repeat length created additional Wt1(–KTS) binding sites, while an increase in AG repeat length created TFII-I and GAGA factor binding sites. If the AG repeat length was reduced, TFII-I sites were also reduced. Wt1(–KTS) is necessary for normal retina formation in mice [87], while TFII-I is a signal-induced multifunctional transcription factor that plays a key role in the regulation of cell proliferation [88]. Moreover, the GAGA factor, a transcription activator, is activated by epidermal growth factors, platelet-derived growth factors, and insulin [89]. These growth factors could regulate *PAX6* transcription through the GAGA factor binding site.

In summary, we found no association between polymorphisms in the *PAX6* coding region and high myopia in our Hong Kong Chinese cohort. Two dinucleotide repeats, AC and AG, in the *PAX6* P1 promoter were associated with high myopia. These two repeats were also associated with the elevation of *PAX6* P1 promoter activity, and hence an increase in the transcriptional activity of *PAX6*. Our results provide evidence for the role of *PAX6* in the pathogenesis of high myopia.

REFERENCES

1. Wong TY, Foster PJ, Hee J, Ng TP, Tielsch JN, Chew SJ, Johnson GJ. Seah SKL. Prevalence and risk factors for refractive errors in adult Chinese in Singapore. Invest Ophthalmol Vis Sci 2000; 41:2486-94. [PMID: 10937558]
2. Kleinstein RN, Jones LA, Hullett S, Kwon S, Lee RL, Friedman NE, Manny RE, Mutti DO, Yu JA, Zadnik K, Collaborative Longitudinal Evaluarion of Ethnicity and Refractive Error Study Group. Refractive error and ethnicity in children. Arch Ophthalmol 2003; 121:1141-7. [PMID: 12912692]
3. Saw SM, Tong L, Chua WH, Chia KS, Koh D, Tan DT, Katz J. Incidence and progression of myopia in Singaporean school children. Invest Ophthalmol Vis Sci 2005; 46:51-7. [PMID: 15623754]
4. Fan DS, Lam DS, Lam RF, Lau JT, Chong KS, Cheung EY, Lai RY, Chew SJ. Prevalence, incidence, and progression of myopia of school children in Hong Kong. Invest Ophthalmol Vis Sci 2004; 45:1071-5. [PMID: 15037570]
5. Lam CS, Goldschmidt E, Edwards MH. Prevalence of myopia in local and international schools in Hong Kong. Optom Vis Sci 2004; 81:317-22. [PMID: 15181356]
6. Saw SM, Shankar A, Tan SB, Taylor H, Tan DT, Stone RA, Wong TY. A cohort study of incident myopia in Singaporean children. Invest Ophthalmol Vis Sci 2006; 47:1839-44. [PMID: 16638989]
7. Mastropasqua L, Lobefalo L, Mancini A, Ciancaglini M, Palma S. Prevalence of myopia in open angle glaucoma. Eur J Ophthalmol 1992; 2:33-5. [PMID: 1638164]
8. Hochman MA, Seery CM, Zarbin MA. Pathophysiology and management of subretinal hemorrhafe. Surv Ophthalmol 1997; 42:195-213. [PMID: 9406367]
9. Lim R, Mitchell P, Cumming RG. Refractive associations with cataract: the Blue Mountains Eye Study. Invest Ophthalmol Vis Sci 1999; 40:3021-6. [PMID: 10549667]
10. Banker AS, Freeman WR. Retinal detachment. Ophthalmol Clin North Am 2001; 14:695-704. [PMID: 11787748]
11. Mutti DO, Mitchell GL, Oeschberge ML, Jones LA, Zadnik K. Parental myopia, near work, school schievment, and children's refractive error. Invest Ophthalmol Vis Sci 2002; 43:3633-40. [PMID: 12454029]
12. Saw SM, Chua WH, Hong CY, Wu HM, Chan WY, Chia KS, Stone RA, Tan D. Nearwork in early-onset myopia. Invest Ophthalmol Vis Sci 2002; 43:332-9. [PMID: 11818374]
13. Hammond CJ, Snieder H, Gilbert CE, Spector TD. Genes and environment in refractive error: the twin eye study. Invest Ophthalmol Vis Sci 2001; 42:1232-6. [PMID: 11328732]
14. Lyhne N, Sjolie AK, Kyvik KO, Green A. The important of genes and environment for ocular refractive and its determiners: a population based study among 20-45 year old twins. Br J Ophthalmol 2001; 85:1470-6. [PMID: 11734523]
15. Dirani M, Chamberlain M, Shekar SN, Islam AF, Garoufalis P, Chen CY, Guymer RH, Baird PN. Heritability of refractive error and ocular biometrics: the Genes in Myopia (GEM) twin study. Invest Ophthalmol Vis Sci 2006; 47:4756-61. [PMID: 17065484]
16. Lopes MC, Andrew T, Carbonaro F, Spector TD, Hammond CJ. Estimating heritability and shared environmental effects for refractive error in twin and family studies. Invest Ophthalmol Vis Sci 2009; 50:126-31. [PMID: 18757506]
17. Angi MR, Clementi M, Sardei C, Piattelli E, Bisantis C. Heritability of myopic refractive errors in identical and fraternal twins. Graefes Arch Clin Exp Ophthalmol 1993; 231:580-5. [PMID: 8224933]

18. Liang CL, Yen E, Su JY, Liu C, Chang TY, Park N, Wu MJ, Lee S, Flynn JT, Jou SH. Impact of family history of high myopia on level and onset of myopia. Invest Ophthalmol Vis Sci 2004; 45:3446-52. [PMID: 15452048]

19. Schwartz M, Haim M, Skarsholm D. X-linked myopia: Bornholm eye disease. Linkage to DNA markers on the distal part of Xq. Clin Genet 1990; 38:281-6. [PMID: 1980096]

20. Young TL, Ronana SM, Drahozal LA, Wildenberg SC, Alvear AB, Oetting WS, Atwood LD, Wilkin DJ, King RA. Evidence that a locus for familial high myopia maps to chromosome 18p. Am J Hum Genet 1998; 63:109-19. [PMID: 9634508]

21. Lam DS, Tam PO, Fan DS, Baum L, Leung YF, Pang CP. Familial high myopia linkage to chromosome 18p. Ophthalmologica 2003; 217:115-8. [PMID: 12592049]

22. Young TL, Ronan SM, Alvear AB, Wildenberg SC, Oetting WS, Atwood LD, Wilkin DJ, King RA. A second locus for familial high myopia maps to chromosome 12q. Am J Hum Genet 1998; 63:1419-24. [PMID: 9792869]

23. Naiglin L, Gazagne C, Dallongeville F, Thalamas C, Idder A, Rascol O, Malecaze F, Calvas P. Genome wide scan for familial high myopia suggests a novel locus on chromosome 7q36. J Med Genet 2002; 39:118-24. [PMID: 11836361]

24. Paluru P, Ronan SM, Heon E, Devoto M, Wildenberg SC, Scavello G, Holleschau A, Makitie O, Cole WG, King RA, Young TL. New locus for autosomal dominant high myopia maps to the long arm of chromosome 17. Invest Ophthalmol Vis Sci 2003; 44:1830-6. [PMID: 12714612]

25. Stambolian D, Ibay G, Reider L, Dana D, Moy C, Schlifka M, Holmes T, Ciner E, Bailey-Wilson JE. Genomewide linkage scan for myopia susceptibility loci among Ashkenazi Hewish families shows evidence of linkage on chromosome 22q12. Am J Hum Genet 2004; 75:448-59. [PMID: 15273935]

26. Hammond CJ, Andrew T, Mak YT, Spector TD. 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 2004; 75:294-304. [PMID: 15307048]

27. Zhang Q, Guo X, Xiao X, Jia X, Li S, Hejtmancik JF. A new locus for autosomal dominant high myopia maps to 4q22-q27 between D2S1578 and D4S1612. Mol Vis 2005; 11:554-60. [PMID: 16052171]

28. Paluru PC, Nallasamy S, Devoto M, Rappaport EF, Young TL. Identification of a novel locus on 2q for autosomal dominant high-grade myopia. Invest Ophthalmol Vis Sci 2005; 46:2300-7. [PMID: 15980214]

29. Zhang Q, Guo X, Xiao X, Jia X, Li S, Hejtmancik JF. Novel locus for X linked recessive high myopia maps to Xq23-q25 but outside MYP1. J Med Genet 2006; 43:e20. [PMID: 16648373]

30. Wojciechowski R, Moy C, Ciner E, Ibay G, Reider L, Bailey-Wilson JE, Stambolian D. Genomewide scan in Ashkenazi Jewish families demonstrates evidence of linkage of ocular refraction to a QTL on chromosome 1p36. Hum Genet 2006; 119:389-99. [PMID: 16501916]

31. Nallasamy S, Paluru PC, Devoto M, Wasserman NF, Zhou J, Young TL. Genetic linkage study of high-grade myopia in a Hutterite population from South Dakota. Mol Vis 2007; 13:229-36. [PMID: 17327828]

32. Lam CY, Tam PO, Fan DS, Fan BJ, Wang DY, Lee CW, Pang CP, Lam DS. A genome-wide scan maps a novel high myopia locus to 5p15. Invest Ophthalmol Vis Sci 2008; 49:3768-78. [PMID: 18421076]

33. Ciner E, Wojciechowski R, Ibay G, Bailey-Wilson JE, Stambolian D. Genomewide scan of ocular refraction in African-American families shows significant linkage to chromosome 7p15. Genet Epidemiol 2008; 32:454-63. [PMID: 18293391]

34. Paget S, Julia S, Vitezica ZG, Soler V, Malecaze F, Calvas P. Linkage analysis of high myopia susceptibility locus in 26 families. Mol Vis 2008; 14:2566-74. [PMID: 19122830]

35. Yang Z, Xiao X, Li S, Zhang Q. Clinical and linkage study on a consanguineous Chinese family with autosomal recessive high myopia. Mol Vis 2009; 15:312-8. [PMID: 19204786]

36. Yu ZQ, Li YB, Huang CX, Chu RY, Hu DN, Shen ZH, Huang W. A genome-wide screening for pathological myopia suggests a novel locus on chromosome 15q12-13. Zhonghua Yan Ke Za Zhi 2007; 43:233-8. [PMID: 17605906]

37. Nishizaki R, Ota M, Inoko H, Meguro A, Shiota T, Okada E, Mok J, Oka A, Ohno S, Mizuki N. New susceptibility locus for high myopia is linked to the uromodulin-like 1 (UMODL1) gene region on chromosome 21q22.3. Eye 2009; 23:222-9. [PMID: 18535602]

38. Wojciechowski R, Stambolian D, Ciner E, Ibay G, Holmes TN, Bailey-Wilson JE. Genomewide linkage scans for ocular refraction and meta-analysis of four populations in the Myopia Family Study. Invest Ophthalmol Vis Sci 2009; 50:2024-32. [PMID: 19151385]

39. Li YJ, Guggenheim JA, Bulusu A, Metlapally R, Abbott D, Malecaze F, Calvas P, Rosenberg T, Paget S, Creer RC, Kirov G, Owen MJ, Zhao B, White T, Mackey DA, Young TL. An international collaborative family-based whole-genome linkage scan for high-grade myopia. Invest Ophthalmol Vis Sci 2009; 50:3116-27. [PMID: 19324860]

40. Schache M, Chen CY, Pertile KK, Richardson AJ, Dirani M, Mitchell P, Baird PN. Fine mapping linkage analysis identifies a novel susceptibility locus for myopia on chromosome 2q37 adjacent to but not overlapping MYP12. Mol Vis 2009; 15:722-30. [PMID: 19365569]

41. Lam DS, Lee WS, Leung YF, Tam PO, Fan DS, Fan BJ, Pang CP. TGFbeta-induced factor: a candidate gene for high myopia. Invest Ophthalmol Vis Sci 2003; 44:1012-5. [PMID: 12601022]

42. Yanovitch T, Li YJ, Metlapally R, Abbott D, Viet KN, Young TL. Hepatocyte growth factor and myopia: genetic association analyses in a Caucasian population. Mol Vis 2009; 15:1028-35. [PMID: 19471602]

43. Hall NF, Gale CR, Ye S, Martyn CN. Myopia and polymorphisms in genes for matrix metalloproteinases. Invest Ophthalmol Vis Sci 2009; 50:2632-6. [PMID: 19279308]

44. Inamori Y, Ota M, Inoko H, Okada E, Nishizaki R, Shiota T, Mok J, Oka A, Ohno S, Mizuki N. The COL1A1 gene and high myopia susceptibility in Japanese. Hum Genet 2007; 122:151-7. [PMID: 17557158]

45. Mutti DO, Cooper ME, O'Brien S, Jones LA, Marazita ML, Murray JC, Zadnik K. Candidate gene and locus analysis of myopia. Mol Vis 2007; 13:1012-9. [PMID: 17653045]

46. Lin HJ, Wan L, Tsai Y, Tsai YY, Fan SS, Tsai CH, Tsai FJ. The TGFβ1 gene codon 10 polymorphism contributes to the genetic predisposition to high myopia. Mol Vis 2006; 12:698-703. [PMID: 16807529]

47. Lin HJ, Wan L, Tsai Y, Liu SC, Chen WC, Tsai SW, Tsai FJ. Sclera-related gene polymorphisms in high myopia. Mol Vis 2009; 15:1655-63. [PMID: 19710942]

48. Lin HJ, Kung YJ, Lin YJ, Sheu JJ, Chen BH, Lan YC, Lai CH, Shu YA, Wan L, Tsai FJ. Association of the Lumican gene functional 3' UTR polymorphism with high myopia. Invest Ophthalmol Vis Sci. 2009 [PMID: 19643966]

49. Khor CC, Grignani R, Ng DP, Toh KY, Chia KS, Tan D, Goh DL, Saw SM. cMET and refractive error progression in children. Ophthalmology 2009; 116:1469-74. [PMID: 19500853]

50. Nishina S, Kohsaka S, Yamaguchi Y, Handa H, Kawakami A, Fujisawa H, Azuma N. PAX6 expression in the developing human eye. Br J Ophthalmol 1999; 83:723-7. [PMID: 10340984]

51. Xu PX, Zhang X, Heaney S, Yoon A, Michelson AM, Maas RL. Regulation of Pax6 expression is conserved between mice and flies. Development 1999; 126:383-95. [PMID: 9847251]

52. Morgan R. Conservation of sequence and function in the Pax6 regulatory elements. Trends Genet 2004; 20:283-7. [PMID: 15219391]

53. Lakowski J, Majumder A, Lauderdale JD. Mechanisms controlling Pax6 isoform expression in the retina have been conserved between teleosts and mammals. Dev Biol 2007; 307:498-520. [PMID: 17509554]

54. Simpson TI, Price DJ. Pax6; a pleiotropic player in development. Bioessays 2002; 24:1041-51. [PMID: 12386935]

55. Hill RE, Favor J, Hogan BL, Ton CC, Saunder GF, Hanson IM, Prosser J, Jordan T, Hastie ND, van Heyningen V. Mouse small eye results from mutations in a paired-like homeobox-containing gene. Nature 1991; 354:522-5. [PMID: 1684639]

56. Kamachi Y, Uchikawa M, Tanouchi A, Sekido R, Kondoh H. Pax6 and SOX2 form a co-DNA-binding partner complex that regulates initiation of lens development. Genes Dev 2001; 15:1272-86. [PMID: 11358870]

57. Plaza S, Dozier C, Turque N, Saule S. Quail Pax-6 (Pax-QNR) mRNAs are expressed from two promoters used differentially during retina development and neuronal differentiation. Mol Cell Biol 1995; 15:3344-53. [PMID: 7760830]

58. Xu ZP, Saunders GF. Transcriptional regulation of the human PAX6 gene promoter. J Biol Chem 1997; 272:3430-6. [PMID: 9013587]

59. Okladnova O, Syagailo YV, Mossner R, Riederer P, Lesch KP. Regulation of PAX-6 gene transcription: alternate promoter usage in human brain. Brain Res Mol Brain Res 1998; 60:177-92. [PMID: 9757029]

60. Okladnova O, Syagailo YV, Tranitz M, Stober G, Riederer P, Mossner R, Lesch KP. A promoter-associated polymorphic repeat modulates PAX-6 expression in human brain. Biochem Biophys Res Commun 1998; 248:402-5. [PMID: 9675149]

61. Glaser T, Jepeal L, Edwards JG, Young SR, Favor J, Maas RL. PAX6 gene dosage effect in a family with congenital cataracts, aniridia, anophthalmia and central nervous system defects. Nat Genet 1994; 7:463-71. [PMID: 7951315]

62. Schedl A, Ross A, Lee M, Engelkamp D, Rashbass P, van Heyningen V, Hastie ND. Influence of PAX6 gene dosage on development: overexpression causes severe eye abnormalities. Cell 1996; 86:71-82. [PMID: 8689689]

63. Favor J, Gloeckner CJ, Neuhauser-Klaus A, Pretsch W, Sandulache R, Saule S, Zaus I. Relationship of Pax6 activity levels to the extent of eye development in the mouse, Mus musculus. Genetics 2008; 179:1345-55. [PMID: 18562673]

64. Simpson CL, Hysi P, Bhattacharya SS, Hammond CJ, Webster A, Peckham CS, Sham PC, Rahi JS. The roles of PAX6 and SOX2 in myopia: lessons from the 1958 British birth cohort. Invest Ophthalmol Vis Sci 2007; 48:4421-5. [PMID: 17898260]

65. Hewitt AW, Kearns LS, Jamieson RV, Williamson KA, van Heyningen V, Mackey DA. PAX6 mutations may be associated with high myopia. Ophthalmic Genet 2007; 28:179-82. [PMID: 17896318]

66. Han W, Leung KH, Fung WY, Mak JY, Li YM, Yap MK, Yip SP. Association of PAX6 polymorphisms with high myopia in Han Chinese nuclear families. Invest Ophthalmol Vis Sci 2009; 50:47-56. [PMID: 19124844]

67. Tsai YY, Chiang CC, Lin HJ, Lin JM, Wan J, Tsai FJA. PAX6 gene polymorphism is associated with genetic predisposition to extreme myopia. Eye 2008; 22:576-81. [PMID: 17948041]

68. Andersson S, Davis DL, Dahlback H, Jornvall H, Russell DW. Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J Biol Chem 1989; 264:8222-9. [PMID: 2722778]

69. Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. Exp Eye Res 1996; 62:155-69. [PMID: 8698076]

70. Sham PC, Curtis D. Monte Carlo tests for association between disease and alleles at highly polymorphic loci. Ann Hum Genet 1995; 59:97-105. [PMID: 7762987]

71. Chen YH, Lin SJ, Lin MW, Tsai HL, Kuo SS, Chen JW, Charng MJ, Wu TC, Chen LC, Ding PY, Pan WH, Jou YS, Chau LY. Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. Hum Genet 2002; 111:1-8. [PMID: 12136229]

72. Rantner B, Kollerits B, Anderwald-Stadler M, Klein-Weigel P, Gruber I, Gehringer A, Haak M, Schnapka-Kopf M, Fraedrich G, Kronenberg F. Association between the UGT1A1 TA-repeat polymorphism and bilirubin concentration in patients with intermittent claudication: Results from the CAVASIC study. Clin Chem 2008; 54:851-7. [PMID: 18375480]

73. Schug J. Using TESS to predict transcription factor binding sites in DNA sequence. In: Baxevanis AD, editor. Current Protocols in Bioinformatics. J. Wiley and Sons; 2003.

74. TESS. Transcription Element Search Software on the WWW. Schug J, Overton GC. Technical Report CBIL-TR-1997-1001-v0.0. Computational Biology and Informatics Laboratory, School of Medicine, University of Pennsylvania, 1997. <http://www.cbil.upenn.edu/tess>.

75. Neethirajan G, Krishnadas SR, Vijayalakshmi P, Shashikant S, Sundaresan P. PAX6 gene variations associated with aniridia in south India. BMC Med Genet 2004; 5:9. [PMID: 15086958]

76. Brown A, McKie M, van Heyningen V, Prosser J. The human PAX6 mutation database. Nucleic Acids Res 1998; 26:259-64. [PMID: 9399848]

77. Grocott T, Frost V, Maillard M, Johansen T, Wheeler GN, Dawes LJ, Wormstone IM, Chantry A. The MH1 domain of Smad3 interacts with Pax6 and represses autoregulation of the Pax6 P1 promoter. Nucleic Acids Res 2007; 35:890-901. [PMID: 17251190]

78. Bhat SP, Rayner SA, Chau SC, Ariyasu RG. Pax-6 expression in posthatch chick retina during and recovery from form-deprivation myopia. Dev Neurosci 2004; 26:328-35. [PMID: 15855761]

79. Zhong XW, Ge J, Deng W, Chen X, Huang J. Expression of pax-6 in rhesus monkey of optical defocus induced myopia form deprivation myopia. Chin Med J 2004; 117:722-6. [PMID: 15161541]

80. Tkatchenko AV, Walsh PA, Tkatchenko TV, Gustincich S, Raviola E. Form deprivation modulates retinal neurogenesis in primate experimental myopia. Proc Natl Acad Sci USA 2006; 103:4681-6. [PMID: 16537371]

81. Sander M, Neubuser A, Kalamaras J, Ee HC, Martin GR, German MS. Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. Genes Dev 1997; 11:1662-73. [PMID: 9224716]

82. Wen JH, Chen YY, Song SJ, Ding J, Gao Y, Hu QK, Feng RP, Liu YZ, Ren GC, Zhange CY, Hong TP, Gao X, Li LS. Paired box 6 (PAX6) regulates glucose metabolism via proinsulin processing mediated by prohormone convertase 1/3 (PC1/3). Diabetologia 2009; 52:504-13. [PMID: 19034419]

83. Feldkaemper MP, Neacsu I, Schaeffel F. Insulin acts as a powerful stimulator of axial myopia in chicks. Invest Ophthalmol Vis Sci 2009; 50:13-23. [PMID: 18599564]

84. Cordain L, Eaton SB, Brand Miller J, Lindeberg S, Jensen C. An evolutionary analysis of the aetiology and pathogenesis of juvenile-onset myopia. Acta Ophthalmol Scand 2002; 80:125-35. [PMID: 11952477]

85. Feldkaemper MP, Schaeffel F. Evidence for a potential role of glucagon during eye growth regulation in chicks. Vis Neurosci 2002; 19:755-66. [PMID: 12688670]

86. Zhu X, Wallman J. Opposite effects of glucagon and insulin on compensation for spectacle lenses in chicks. Invest Ophthalmol Vis Sci 2009; 50:24-36. [PMID: 18791176]

87. Wagner KD, Wagner N, Vidal VP, Schley G, Wilhelm D, Schedl A, Englert C, Scholz H. The Wilms' tumor gene Wt1 is required for normal development of the retina. EMBO J 2002; 21:1398-405. [PMID: 11889045]

88. Roy AL. Signal-induced functions of the transcription factor TFII-I. Biochim Biophys Acta 2007; 1769:613-621. [PMID: 17976384]

89. Wyse BD, Linas SL, Thekkumkara TJ. Functional role of a novel cis-acting element (GAGA box) in human type-1 angiotensin II receptor gene transcription. J Mol Endocrinol 2000; 25:97-108. [PMID: 10915222]