Mutational Analysis of *MIR184* in Sporadic Keratoconus and Myopia

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Purpose. A mutation miR-184(+57C>T) in the seed region of miR-184 (encoded by *MIR184* [MIM*613146]) results in familial severe keratoconus combined with early-onset anterior polar cataract and endothelial dystrophy, iris hypoplasia, congenital cataract, and stromal thinning (EDICT) syndrome (MIM#614303). In order to investigate the phenotypic spectrum resulting from *MIR184* mutation, *MIR184* was sequenced in a keratoconus cohort of mixed ethnicity and a Chinese axial myopia cohort.

Methods. Sequencing of *MIR184* was performed in 780 unrelated keratoconus patients and 96 unrelated Han southern Chinese subjects with axial myopia. Effects of identified mutations on RNA secondary structure were predicted computationally using mFold and RNAFold algorithms. *MIR184* amplicons from patients harboring mutations were cloned and transfected into human embryonic kidney 293T (HEK293T) cells, and mature mutant miR-184 expression was analyzed by stem-loop real-time quantitative PCR (RT-qPCR).

Results. Two novel heterozygous substitution mutations in *MIR184* were identified in the two patients with isolated keratoconus: miR-184(+8C>A) and miR-184(+3A>G). Computational modeling predicted that these mutations would alter the miR-184 stem-loop stability and secondary structure. Ex vivo miR-184 expression analysis demonstrated that miR-184(+8C>A) almost completely repressed the expression of miR-184 ($P = 0.022$), and miR-184(+3A>G) reduced the expression of miR-184 by approximately 40% ($P = 0.002$). There was no significant association of rs41280052, which lies within the stem-loop of miR-184, with keratoconus. No *MIR184* mutations were detected in the axial myopia cohort.

Conclusions. Two novel heterozygous substitution mutations in *MIR184* were identified in two patients with isolated keratoconus: miR-184(+8C>A) and miR-184(+3A>G). Mutations in *MIR184* are a rare cause of keratoconus and were found in 2 of 780 (0.25%) cases.

Keywords: keratoconus, myopia, MIR184, hsa-miR-184, corneal dystrophies, hereditary

Keratoconus (MIM#148300), a common disorder of the corneal shape and structure,[1] is the leading indication for corneal transplantation in the developed world.[2] Clinically, keratoconus appears as a bilateral, noninflammatory progressive corneal ectasia in which the cornea protrudes and thins, manifesting as progressive myopia and irregular astigmatism.[1,3] Clinically, the severity of keratoconus ranges from the mild subclinical forme fruste keratoconus to myopia and irregular astigmatism to severe progressive conical protrusion, scarring, or blindness.[1,3] Keratoconus is a lifelong condition that is a significant health burden in work-age adults, affecting quality of life.[4] Despite the visual and social impact of keratoconus,[5,6] the underlying biochemical processes and pathobiology remain poorly understood.[1]

There is strong evidence that keratoconus has a genetic basis,[1,7–11] but to date, few if any genes have been identified. Mutations in the visual system homeobox gene 1 (*VSX1*; MIM*605020) on 20p11.2 (KTCN1; MIM#148300) have been described in keratoconus.[12] However, this has proved a controversial finding,[13] and further studies excluded sequence changes in the *VSX1* gene as the cause of disease in their patient populations,[14–16] demonstrating that *VSX1* is not a major cause of keratoconus. In the superoxide dismutase 1 gene (*SOD1*; MIM*147450), a heterozygous 7-bp deletion in intron 2 (IVS 2+50 del 7) was identified in two families with keratoconus,[17] but other groups have failed to detect *SOD1* mutations in their patient cohorts.[16,18] Genome-wide association studies (GWAS) have been conducted in keratoconus cohorts and identified SNPs in the hepatocyte growth factor (*HGF*; MIM*142409),[19] RAB3 GTPase activating subunit 1 (*RAB3GAP1*; MIM*602536),[20] and lysyl oxidase (*LOX*; MIM*153455)[21] associated with keratoconus susceptibility.

We recently identified a mutation miR-184(+57C>T) in the seed region of miR-184 (encoded by *MIR184* [MIM*613146]) responsible for familial severe keratoconus, combined with early-onset anterior polar cataract, by targeted resequencing of a 5.5-Mb linkage region at 15q22-q24 known to contain the mutation.[22] A second identical mutation in *MIR184* has been recently reported in a family with endothelial dystrophy, iris hypoplasia, congenital cataract, and stromal thinning (EDICT) syndrome (MIM#614303).[23,24] Axial myopia is associated with keratoconus, and keratoconic eyes have on average longer axial and posterior segment lengths than emmetropic eyes.[25] In order to investigate the phenotypic spectrum resulting from *MIR184* mutation and potential genotype–phenotype relationships, we sequenced *MIR184* in a keratoconus cohort of mixed ethnicity and a Chinese cohort with axial myopia. A known SNP, rs41280052, located within the pre-miR-184 sequence was investigated to identify a possible association with keratoconus.

Methods

Patients

Clinically affected keratoconus patients were recruited as part of ongoing studies from Belfast (Belfast Health and Social Care Trust, UK), Leeds (St. James's University Hospital, Leeds, UK), Australia (Flinders University, Adelaide, Australia), and India (Aravind Eye Hospital, Madurai, India). The diagnosis of keratoconus was performed by an experienced ophthalmologist based on well-established clinical signs on slit-lamp biomicroscopy, cycloplegic retinoscopy, and corneal topography. Patients with at least one clinical sign of the disease in conjunction with a confirmatory corneal topography map were considered as having keratoconus.

A cohort from a Han Chinese population with axial myopia was recruited as part of ongoing myopia genetics studies[26–28] at The Hong Kong Polytechnic University. Myopic subjects were excluded if they showed obvious signs of ocular disease or other inherited disease associated with myopia.

All studies adhered to the tenets of Declaration of Helsinki and were approved by the relevant institutional review boards, with all participants giving written informed consent.

PCR Amplification and DNA Sequencing

Genomic DNA was extracted from peripheral blood leukocytes from all subjects using commercial kits according to the manufacturer's instructions. PCR primers for amplification of the *MIR184* stem-loop sequence (available in the public domain in miRBase at http://www.mirbase.org/; MI0000481) and flanking regions were designed using Primer3 Detective V1.01 program (available in the public domain at http://frodo.wi.mit.edu/primer3/)[29]: miR-184F 5′-CCAGGTGTCAGAGGGAGAGA-3′ and miR-184R 5′-CCAAGGTCTCCTCCTGGAAT-3′. Sanger sequencing of *MIR184* was performed (conditions available on request), and the sequencing results were analyzed manually using the sequence analysis software SeqScape version 2.1.1 (Applied Biosystems, Foster City, CA). Mutations were annotated in accordance with the sequence presented in miRBase (MI0000481). Secondary structural effects of identified mutations were assessed computationally using mFold (available in the public domain at http://mfold.rna.albany.edu/?q=mfold)[30] and RNAFold algorithms (available in the public domain at http://www.tbi.univie.ac.at/RNA/index.html). The conservation of the nucleotides across species was analyzed using the UCSC genome browser (available in the public domain at http://genome.ucsc.edu/).

Ex Vivo miR-184 Expression Analysis

MIR184 amplicons from patients harboring mutations were gel purified using a GeneJET Gel Extraction Kit (Fermentas, Loughborough, UK) following manufacturer's instructions and cloned into Vivid Colors pcDNA 6.2/N-EmGFP-GW/TOPO Mammalian Expression Vector (Life Technologies, Paisley, UK) following the manufacturer's protocol. The resulting recombinant plasmids containing inserts with mutations in *MIR184* and wild-type (WT) controls were transfected into human embryonic kidney 293T (HEK293T) cells from the American Type Culture Collection (ATCC, LGC Standards, Teddington, Middlesex, UK). The cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum and 100 μg/mL Primocin (InvivoGen; Source BioScience LifeSciences, Nottingham, UK). Transfections were performed using TurboFect Transfection Reagent (Fermentas) according to the manufacturer's protocol. At least three independent biological replicates were performed for each transfection (untransfected control: $n = 3$; WT: $n = 3$; miR-184(+3G>A): $n = 6$; miR-184(+8C>A): $n = 3$). RNA was extracted from the cells 20 hours after transfection using miRNeasy Mini Kit (Qiagen, Manchester, UK) according to the manufacturer's instructions.

Real-Time Quantitative PCR (RT-qPCR)

RT-qPCR was performed using TaqMan MicroRNA assays (hsa-miR-184 [000485], hsa-miR-103 [000439], hsa-miR-218 [000521]; Life Technologies) according to manufacturer's instructions with 5 ng total RNA. For each biological replicate, three PCR technical replicates were performed. Reactions were amplified using the LightCycler 480 Real-Time PCR System (Roche Diagnostics Corporation, Indianapolis, IN) (conditions available on request). Cycle threshold (Ct) values were calculated using LightCycler 480 software 1.5 (Roche Diagnostics Corporation); then differences in expression were analyzed with the relative expression software tool (REST).[31] Differences in expression were considered statistically significant at $P < 0.05$.

rs41280052 SNP Genotyping Assay

The genotype of SNP rs41280052 in 692 Caucasian keratoconus cases was determined through the sequencing of the *MIR184* gene. Unaffected individuals ($n = 1865$) were obtained from the Blue Mountains Eye Study. All were Caucasians older than 49 years of age and had had an ophthalmological examination; the recruitment of this cohort has been described

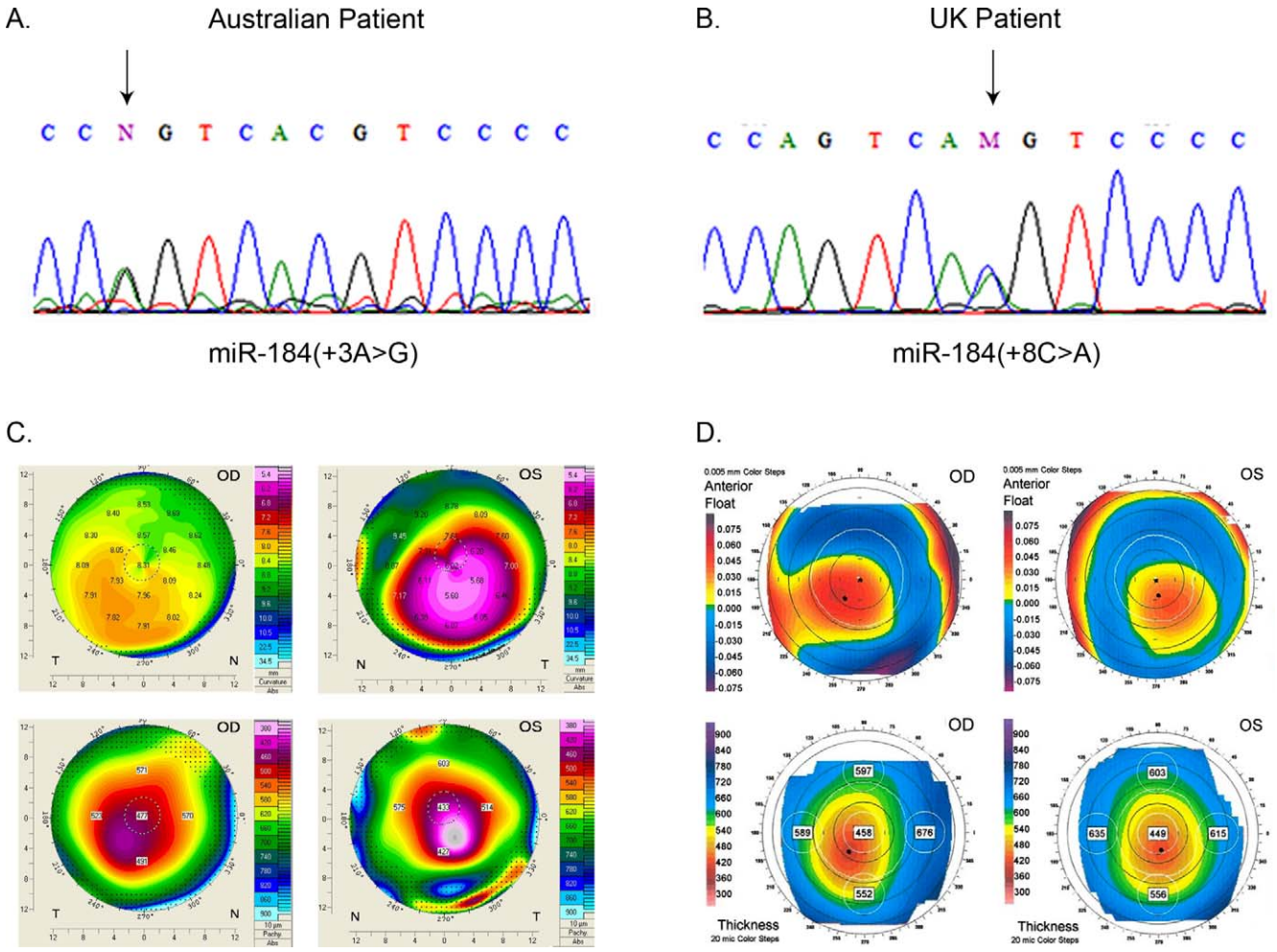

Figure 1. Sequence chromatograms showing the novel heterozygous substitution mutation miR-184(+3A>G) detected in a keratoconus patient from the Australian cohort (**A**) and miR-184(+8C>A) detected in a keratoconus patient from the UK cohort (**B**); corneal topography maps confirming typical keratoconus pattern with anterior corneal steepening (*top*) and corneal thinning (*bottom*) in the right (OD) and left (OS) eye of the Australian patient carrying miR-184(+3A>G) using Pentacam (**C**) and of the UK patient carrying miR-184(+8C>A) using Orbscan II (**D**).

previously.[32] Unaffected controls were genotyped using a custom-designed Taqman SNP genotyping assay and Taqman SNP Genotyping Master Mix performed according to manufacturer's protocols on a StepOnePlus Real-Time PCR instrument (Life Technologies, Mulgrave, Victoria, Australia) (available in the public domain at https://www5.appliedbiosystems.com/tools/genotyping/). Association of the SNP with keratoconus was assessed by χ^2 test.

Results

MiR-184 Sequencing

A total of 780 unrelated keratoconus patients were recruited: 667 of European Caucasian and 123 of South Indian origin. In addition, 96 patients with axial myopia (mean spherical error −9.75 diopters, mean cylindrical error −1.31 diopters, mean spherical equivalent −10.41 diopters, mean axial length 27.60 mm) and 96 controls (spherical equivalent within ±1.0 diopters) were recruited in Hong Kong and were all unrelated Han Chinese. No sequence variants were identified in *MIR184* in the myopia subjects. Two novel heterozygous substitution mutations in *MIR184* were identified in two patients from the keratoconus cohort: miR-184(+3A>G) and miR-184(+8C>A) (Fig. 1). Both patients were Caucasian, one from Australia [miR-184(+3A>G)] and one from the United Kingdom [miR-184(+8C>A)]. Both variants were absent from ethnically matched controls without ocular disease (96 UK and 96 Australian controls) and the 1000 Genomes Project data, release 12, May 2012 (available in the public domain at http://browser.1000genomes.org/index.html; accessed July 2012). The mutation miR-184(+8C>A) was identified in the NHLBI GO Exome Sequencing Project dataset (Exome Variant Server, NHLBI Exome Sequencing Project [ESP]; Seattle, WA [available in the public domain at http://evs.gs.washington.edu/EVS/; accessed July 2012]) with a minor allele frequency of 0.01%, while miR-184(+3A>G) was not present in the ESP data. The cytosine at position 8 of pre-miR-184 is highly conserved across all species; the adenine at position 3 is well conserved, although a guanine is present in some rodents (Fig. 2).

Clinical Details

The UK patient [miR-184(+8C>A)] was a 43-year-old male with a Snellen best-corrected visual acuity of 20/32 (right eye) and 20/20 (left eye) with contact lenses. On slit-lamp biomicroscopy there was definite keratoconus, and Orbscan II (Bausch & Lomb, Salt Lake City, UT) topography confirmed keratoconus (Fig. 1). The central corneal thickness was 458 μm (right eye)

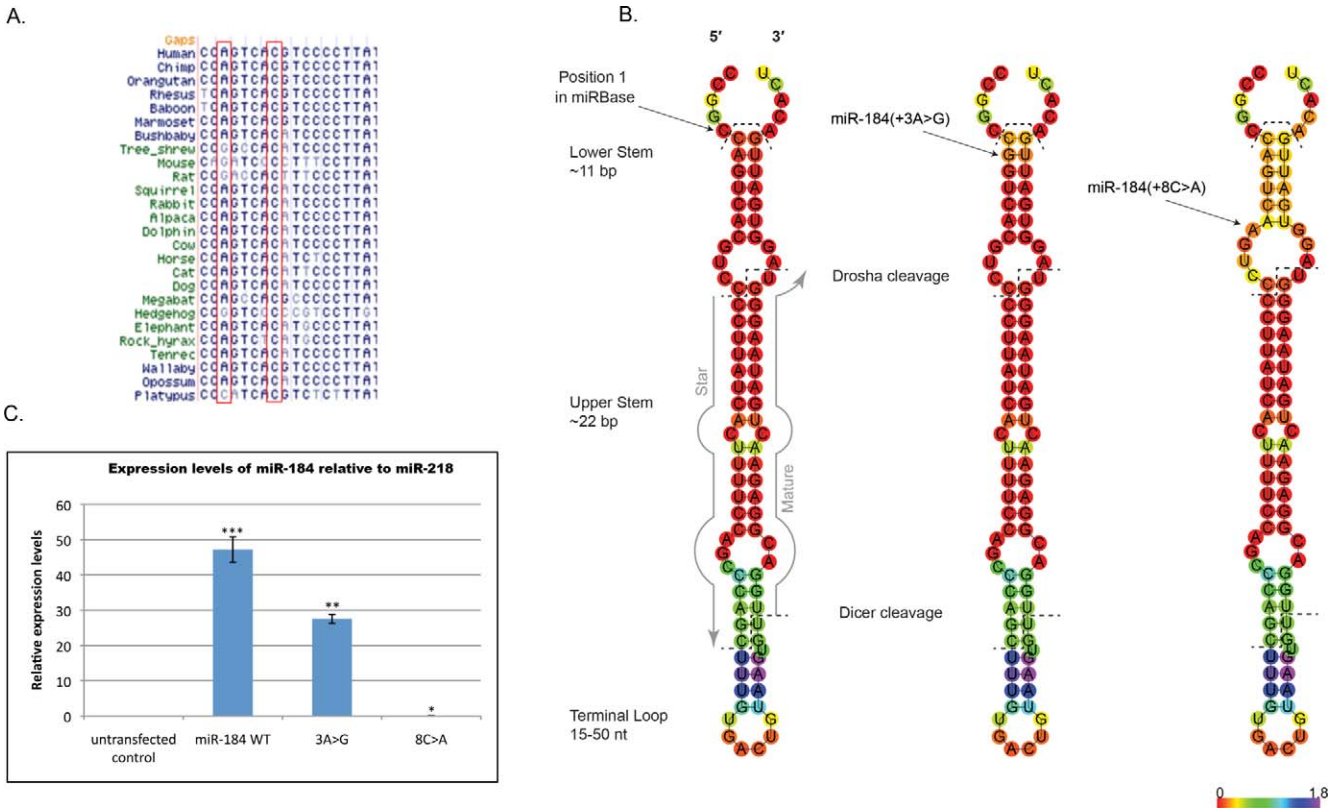

Figure 2. (**A**) Adenine at position 3 of miR-184 is well conserved across mammals, and cytosine at position 8 is well conserved across all species; (**B**) the identified mutations were predicted to cause structural changes of the stem-loop precursor of miR-184. *Colors* represent positional entropy. *Left to right*: miR-184 WT, 3A>G and 8C>A; (**C**) relative expression levels of miR-184 were significantly decreased after transfection with mutant transcripts compared to transfection with WT miR-184 transcripts. (Average relative expression of biological replicates and standard deviations are shown; untransfected control: $n = 3$; WT: $n = 3$; miR-184(+3A>G): $n = 6$; miR-184(+8C>A): $n = 3$.) $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

and 449 μm (left eye). Keratometry measurements were 52.3/49.1 diopters (D) (right eye) and 49.3/46.7 D (left eye). There was no evidence of anterior segment dysgenesis; there were no iris or lenticular abnormalities, and the fundi were normal. The female sibling of the proband had a normal ocular examination including corneal topography and had a WT miR-184 sequence. DNA was available from one elderly infirm parent who carried the same mutation (+8C>A) but was unable to undergo a clinical examination (Fig. 3). Historically there was no mention of a diagnosis of keratoconus in this parent, but keratoconus or forme fruste could not be excluded.

The Australian patient [miR-184(+3A>G)] was a 55-year-old male with bilateral keratoconus with a Snellen best-corrected visual acuity of 20/20 (right eye) and 20/32 (left eye) with contact lenses. Slit-lamp biomicroscopy showed definite keratoconus with anterior stromal scarring on the apex of the cone, prominent corneal nerves, and unique cortical granular cataract. There were no iris abnormalities or anterior segment dysgenesis, and the fundi were normal. Pentacam (Oculus, Wetzlar, Germany) topography confirmed keratoconus (Fig. 1). The central corneal thickness was 497 μm (right eye) and 385 μm (left eye). Keratometry measurements were 41.7/41 D (right eye) and 58/50.5 D (left eye). The patient had an affected brother who carried the same mutation. This patient had bilateral keratoconus with a Snellen best-corrected visual acuity of 20/32 (right eye) and 20/20 (left eye) with contact lenses. Slit-lamp biomicroscopy showed definite keratoconus and very subtle cortical granular cataracts. There were no iris abnormalities or anterior segment dysgenesis, and the fundi were normal. Pentacam (Oculus) topography confirmed keratoconus. The central corneal thickness was 378 μm (right eye) and 408 μm (left eye). Keratometry measurements were 48.2/46.5 D (right eye) and 45.0/44.6 D (left eye).

Examination of the parents in the Australian family (Fig. 3) revealed that neither parent had keratoconus (K readings: mother: right eye 42.75/42.75 D, left eye 43.0/42.75 D; father: right eye 43.0/41.87 D, left eye 42.75/42.37 D). Both parents had thin central corneas (mother: right eye 480 μm, left eye 473 μm; father: right eye 509 μm, left eye 521 μm). The mother displayed prominent corneal nerves and a granular cortical cataract similar to that observed in the brothers. The father had central corneal haze and a Hudson-Stahli iron line. Sequencing of the *MIR184* gene in the parents revealed that the father carried the same mutation (+3A>G). Therefore, the cataract observed in the mother and her two sons was not related to this mutation. As neither parent displayed keratoconus, this mutation appears to be associated with reduced penetrance in the father, or alternatively is not the causative mutation in this family.

MiR-184 Modeling

In order to model the effects of these mutations on the miR-184 stem-loop stability and secondary structure, we used the mFold[30] and RNAFold algorithms. The minimum free energy prediction (ΔG) for WT miR-184 was ΔG = −35.50 kcal/mol compared to miR-184(+3A>G) with ΔG = −34.70 kcal/mol and miR-184(+8C>A) with ΔG = −33.40 kcal/mol (Fig. 2). Both mFold and RNAFold algorithms predicted reduced stability for miR-184(+3A>G) and miR-184(+8C>A), indicated by a change in Gibbs free energy (ΔG) of +0.8 kcal/mol and +2.1 kcal/mol, respectively. While the Gibbs free energy predictions of the

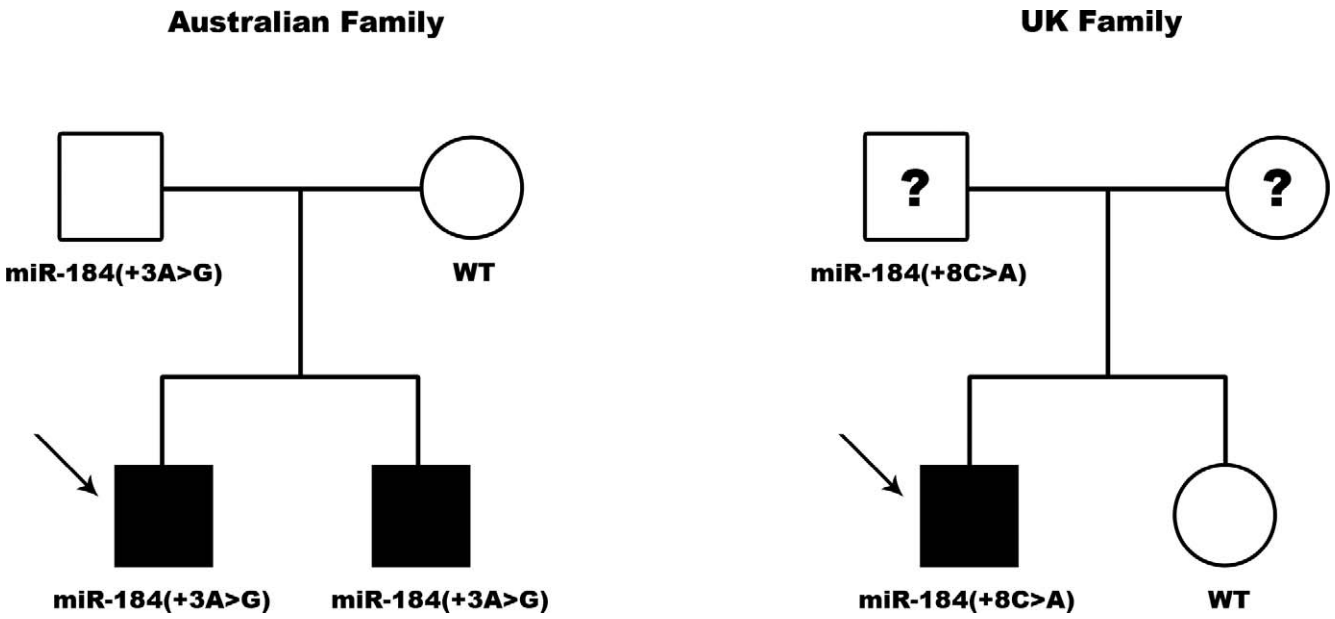

FIGURE 3. Pedigree diagrams of the Australian family (*left*) and UK family (*right*). The proband of each family is indicated by an *arrow*.

mutant miR-184 sequences lie within the normal range (e.g., Gibbs free energy prediction of miR-100 = −25.7 and of miR-183 = −40.50), this predicted reduced stability of miR-184 could affect the processing of the microRNA. Both mutations miR-184(+3A>G) and miR-184(+8C>A) alter the stem-loop secondary structure of miR-184. The mutation at position 3 changes an adenine residue, which normally binds to a uracil in the WT miR-184 stem-loop structure, to a guanine residue, resulting in a reduced base pair probability. For the mutation at position 8, substitution of a cytosine residue, which normally binds guanine in the WT miR-184 stem-loop structure, with an adenine residue increases a bulge of nonpaired residues from six to eight (demonstrated in Fig. 2).

Ex Vivo miR-184 Expression Analysis

Expression levels of mature miR-184 were analyzed by stem-loop RT-qPCR relative to miR-103 (data not shown) and miR-218 expression levels (Fig. 2). Both reference microRNAs showed constant expression in all samples and delivered comparable results. As expected, untransfected HEK293T did not express miR-184, and a statistically significant increase in miR-184 expression was observed after transfection with WT miR-184 ($P < 0.001$). Transfection of miR-184(+3A>G) and miR-184(+8C>A) showed statistically significant reduction of miR-184 levels compared to transfected WT miR-184 ($P = 0.002$ and $P = 0.022$, respectively). While miR-184(+8C>A) almost completely repressed the expression of miR-184, miR-184(+3A>G) reduced the expression of miR-184 by approximately 40%.

SNP Association

A total of 692 keratoconus patients (1384 chromosomes) and 1865 control individuals (3730 chromosomes) were genotyped for SNP rs41280052, which lies within the stem-loop of miR-184. In the keratoconus group, 1366 chromosomes carried the major allele (G) and 18 carried the minor allele (T), resulting in a minor allele frequency of 1.3%. For the control group, 3668 chromosomes carried the major allele and 62 chromosomes carried the minor allele, resulting in a minor allele frequency of 1.7%. A Pearson's χ^2 test gave a χ^2 value of 0.86, 1 degree of freedom (d.f.), P value = 0.3537. Hence, no significant association with keratoconus was observed for rs41280052. The minor allele frequencies for rs41280052 reported on dbSNP (Build 137) were 0.6% (1000 Genomes Project data) and 2.1% (ClinSeq project).[33]

DISCUSSION

MicroRNAs (miRNA) are small (19–25 nucleotides), single-stranded noncoding RNAs that are important regulators of eukaryotic gene expression in most biological processes.[34–36] They act by guiding the RNA-induced silencing complex (RISC) to partially complementary sequences in target mRNAs, mainly in the 3′-untranslated region (3′UTR), to suppress gene expression by a combination of translational inhibition and mRNA decay. Nucleotides two to eight at the 5′ end of the mature miRNA form the seed sequence and are the most important residues in determining mRNA target sites. Transcription of miRNAs in the nucleus results in long transcripts known as primary miRNA transcripts (pri-miRNA), which are processed and cleaved by the RNase III enzyme Drosha into smaller, approximately 70-nucleotide stem-loop miRNA precursors (pre-miRNA). Pre-miRNAs are exported into the cytoplasm by the RanGTP-dependent Exportin-5, where they are further cleaved by another RNase III enzyme, Dicer, to produce the 19- to 25-nucleotide-long miRNA duplex. Only one strand, the mature miRNA, is incorporated into the miRNA-induced silencing complex (miRISC) while the other strand, known as miRNA-star (miRNA*), is degraded. Within the miRISC complex, miRNAs then bind to their target mRNAs to regulate gene expression.[34–36] The structures of miRNA precursors are crucial for recognition and cleavage by Drosha and Dicer proteins during miRNA processing.[37] Structural changes induced by mutations are likely to interfere with the processing of miRNAs, altering miRNA expression or modifying downstream processes and pathways.

The first example of point mutations in a miRNA involved in human disease was reported in 2009 by Mencia et al., who identified two mutations in the seed region of *MIR96* in two Spanish families affected by nonsyndromic progressive hearing loss.[38] We recently reported a mutation [miR-184(+57C>T)] in

the seed region of *MIR184* in a family with severe keratoconus and anterior polar cataract. Subsequently, a second identical mutation in *MIR184* was reported in a family with EDICT syndrome.[23,24] At this time we are unable to confirm whether the *MIR184* mutation arose independently or whether the families are distantly related. The corneal phenotype in EDICT syndrome was described as nonectatic thinning with a uniform corneal steepening on corneal topography.[39,40] That is, the corneal phenotype was not typical of keratoconus clinically or histologically.[39,40] The Northern Irish family in whom we identified the *MIR184* mutation [miR-184(+57C>T)][22] had clinical and topographic features of severe keratoconus.[39] In order to determine the role of *MIR184* mutations in the development of keratoconus, we sequenced *MIR184* in a large keratoconus cohort of mixed ethnicity from the United Kingdom, India, and Australia. Our data expand the current phenotypic spectrum for *MIR184* mutations, which ranges from keratoconus to keratoconus associated with cataract[22,41] to complete anterior segment dysgenesis.[23,24,40] Screening of *MIR184* in a cohort of 96 myopia patients from Hong Kong did not reveal any mutations, indicating that in this patient population, *MIR184* does not play a major role in myopia pathogenesis.

We detected two novel heterozygous mutations in *MIR184* in two keratoconus patients, both of Caucasian origin: miR-184(+3A>G) and (+8C>A). These patients had definitive evidence of keratoconus on slit-lamp examination and corneal topography, indicating the role of *MIR184* mutation in the pathogenesis of isolated keratoconus. Given that the penetrance of the miR-184(+3A>G) mutation is not complete and that we were unable to verify segregation of the miR-184(+8C>A) mutation, computational modeling and functional assays were employed to confirm the pathogenicity of the identified mutations. Computational analysis of the identified mutations in *MIR184* predicted structural changes altering the stem-loop secondary structure of pri-miR-184, as indicated by changes in Gibbs free energy of mutant forms of miR-184 compared to WT miR-184. The structure of the precursor miRNA is crucial for Drosha cleavage and Exportin-5 recognition as well as for Dicer recognition and cleavage specificity. The identified mutations, modifying the stem-loop precursor structure of miR-184, could interfere with both the efficiency of processing and the site of cleavage by Drosha. Changes in the cleavage site would alter the ends of the miR-184 pre-miR and therefore potentially affect Exportin-5 recognition or Dicer processing, resulting in altered expression levels of the mature microRNA. A modification in the Dicer cleavage position would produce a different mature miRNA sequence[37] with an altered seed region.

Cloning and transfection of mutant *MIR184* transcripts into HEK293T cells resulted in a decrease in mature miR-184 expression levels compared to transfected miR-184 WT transcripts, indicating altered miRNA processing. The miR-184(+8C>A) mutation identified in the UK patient was predicted to increase the size of an internal loop close to the hairpin base and resulted in a complete loss of miR-184 expression. The miR-184(+3A>G) mutation was predicted to reduce the base pair probability at position 3 of the miRNA stem-loop structure in the junction region between single- and double-stranded RNA. This region is essential for recognition and cleavage by Drosha,[37] and may be disrupted by the mutation at position 3 of the miR-184 stem-loop structure, resulting in the observed decreased expression level of miR-184. The impact of the miR-184(+3A>G) mutation on the level of miR-184 expression was less than that observed for the miR-184(+8C>A) (Fig. 2), consistent with the incomplete penetrance of this mutation in the Australian family. The penetrance of the miR-184(+3A>G) mutation is not complete, given that the carrier parent did not manifest keratoconus under the commonly used definitions, nor was forme fruste keratoconus evident. However, both parents have some subtle features that are often part of the keratoconus description (thin corneas, corneal haze, prominent corneal nerves). Thus, we hypothesize that the mother, while not harboring a *MIR184* mutation, likely carries other undefined keratoconus genetic risk factors that have been inherited by her offspring. These factors, in conjunction with the *MIR184* mutation, have led to overt keratoconus in the proband and his brother. This polygenic threshold model of disease has long been proposed in complex disease and has been explored extensively in relation to disorders such as schizophrenia.[42]

It is estimated that approximately 60% of human protein coding genes are regulated by miRNAs.[35] One miRNA can target hundreds of downstream target mRNAs, while one mRNA can be targeted by multiple miRNAs.[35] Mutations in the mature miR-184 sequence would alter mRNA target specificity, leading to a cascade of downstream effects on gene expression. Mutations that reduce the expression of mature miR-184 would reduce the regulatory effect upon its target genes. Messenger RNAs targeted by miRNAs can be predicted in silico using a range of algorithms[43] but require functional characterization. In epithelia, miR-184 competitively inhibits the binding of miR-205, encoded by *MIR205* (MIM*613147), to mRNA of the inositol polyphosphate phosphatase-like 1 gene (*INPPL1*; MIM*600829). Functionally, we demonstrated that the miR-184 mutant fails to compete with miR-205 for overlapping target sites on the 3′UTRs of *INPPL1* and *ITGB4*.[22] There is biological evidence that both *INPPL1* and *ITGB4* are involved in the pathogenesis of keratoconus.[22] Recently, miR-184 was shown to regulate the differentiation of human-induced pluripotent stem cells into corneal epithelial-like cells. Knockdown of miR-184 caused a decrease in paired box gene 6 (*PAX6*; MIM*607108), a major regulator of eye development, and keratin 3 (K3; MIM*148043) expression during differentiation.[44] Identification of miR-184 target genes and regulatory pathways may explain the phenotypic spectrum observed in patients harboring miR-184 mutations, but more importantly will assist in the identification of new keratoconus susceptibility genes.

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