SCIENTIFIC REPORT

Development of a DNA chip for the diagnosis of the most common corneal dystrophies caused by mutations in the βigh3 gene

So Young Yoo, Tae-Im Kim, Sang Yup Lee, Eung Kweon Kim, Ki Chang Keum, Nae Choon Yoo, Won Min Yoo

Br J Ophthalmol 2007;**91**:722–727. doi: 10.1136/bjo.2006.111070

Aim: To develop a diagnostic DNA chip to detect mutations in the βigh3 gene causing the most common corneal dystrophies (CDs).

Methods: Samples from 98 people, including patients with βigh3-associated CDs (β-aCDs), were examined. Specific primer and probe sets were designed to examine exons 4 and 12 of the βigh3 gene, in order to identify mutant and wild-type alleles. Mutations were then identified by hybridisation signals of sequence-specific probes immobilised on the slide glass.

Results: Direct sequencing of exons 4 and 12 of the βigh3 gene in the patients' genome showed that β-aCDs could be mainly classified into five types: homozygotic Avellino corneal dystrophy (ACD), heterozygotic ACD, heterozygotic lattice CD I, heterozygotic Reis–Bucklers CD and heterozygotic granular CD. Blind tests were performed by applying the target DNA amplified from the genomic DNA isolated from the peripheral blood of the participants onto a DNA chip. The results obtained by DNA chip hybridisation matched well with the direct DNA sequencing results.

Conclusions: The DNA chip developed in this study allowed successful detection of β-aCDs with a sensitivity of 100%. Mutational analysis of exons 4 and 12 of the βigh3 gene, which are the mutational hot spots causing β-aCDs, can be successfully performed with the DNA chip. Thus, this DNA chip-based method should allow a convenient, yet highly accurate, diagnosis of β-aCDs, and can be further applied to diagnose other types of CDs.

Genetic analyses of most common βigh3-associated corneal dystrophies (β-aCDs) such as Avellino corneal dystrophy (ACD), Reis–Bucklers CD (RBCD), lattice CD type I (LCD I) and granular CD (GCD) have shown that most cases are caused by amino acid substitutions within the βigh3 gene. In the case of ACD, the 124th amino acid arginine of the βigh3 protein is substituted by histidine. Similarly, LCD I is caused by R124C, whereas RBCD is caused by R124L. GCD (also called GCD I) is caused by R555W.[1–3] Thus, molecular genetic analysis of the mutations in the βigh3 gene is most adequate to diagnose patients having different types of β-aCDs, in particular those with an atypical or ambiguous corneal appearance.[4–6]

The molecular genetic analysis of the mutations in the βigh3 gene has mainly relied on direct sequencing. However, DNA microarray-based detection of point mutations can be a much more efficient alternative. To analyse the point mutation in a gene by direct sequencing, the DNA fragment containing a mutated site is amplified and extracted with high purity before sequencing. Even when using the automatic DNA sequencer, each signal peak should be checked directly from the raw data to find just a single gene mutation. This sequential work is rather cumbersome and takes 24–48 h. DNA microarray-based assay can be a simple but powerful alternative, as it allows simultaneous handling of multiple samples and sequences[7] and requires only a small amount of the amplified product that can be directly applied onto a DNA chip without any purification step. It takes no more than 6–8 h, and analysis can be performed simply by checking the signal on the expected probe spot regions. In this paper, we report the development of a DNA chip for detection of the mutations in the βigh3 gene, which allows rapid, convenient and accurate diagnosis of β-aCDs.

MATERIALS AND METHODS

Patients and collection of samples

This study was performed in accordance with the Helsinki Agreement, and all participants gave their informed consent before the test, which was approved by Severance Hospital IRB Committee (No 4-2006-0075).

DNA sequence analysis

DNA was extracted from the peripheral blood of participants with the JETQUICK Blood & Cell Culture DNA Spin Kit (GENOMED GmbH, Löhne, Germany). Exons 4 and 12 of the βigh3 gene were amplified by PCR with origin of replication (Ori) 4 primer sets and Ori 12 primer sets, respectively (table 1 and fig 1). The PCR was conducted with a PCR thermal cycler T1 (BioMetra GmbH, Gottingen, Germany). The amplified products were purified with a PCR purification kit (Qiagen, Hilden, Germany). For the verification of mutations and identification of the mutation types, the DNA isolated from all the 98 participants were also sequenced with an automatic

Table 1 Primers used in this study

Primer name	Sequence (5′→3′)
Ori 4 Forward	CCCCAGAGGCCATCCCTCCT
Ori 4 Reverse	CCGGGCAGACGGAGGTCATC
Ori 12 Forward	CATTCCAGTGGCCTGGACTCTACTATC
Ori 12 Reverse	GGGGCCCTGAGGGATCACTACTT
ACD Forward	AGCCCTACCACTCTCAA
ACD Reverse	CAGGCCTCGTTGCTAGGG

ACD, Avellino corneal dystrophy; Ori, origin of replication.

Abbreviations: ACD, Avellino corneal dystrophy; β-aCD, βigh3-associated corneal dystrophy; CD, corneal dystrophy; FITC, fluorescein isothiocyanate; GCD, granular CD; LCD I, lattice CD type I; Ori, origin of replication; RBCD, Reis–Bucklers CD; SSPE, saline/sodium phosphate/EDTA

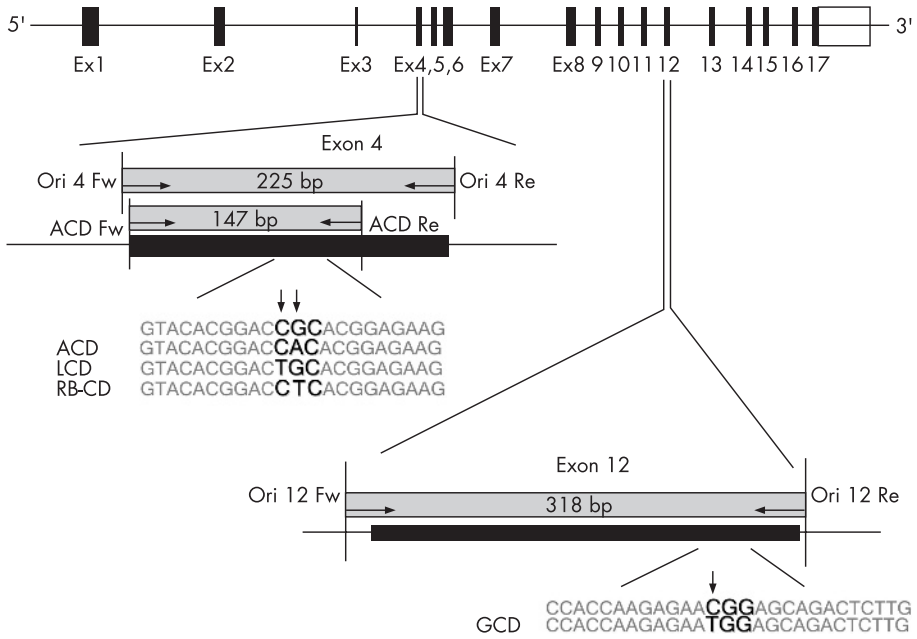

Figure 1 Gene maps and haplotypes of the βigh3 gene. Coding exons are marked by black bars. PCR primers used for the amplification of the targets and the amplified products are shown. Arrows indicate the mutational hot spots in exons 4 and 12 of the common βigh3-associated corneal dystrophies. ACD, Avellino corneal dystrophy; Fw, forward; GCD, granular corneal dystrophy; LCD, lattice corneal dystrophy; Ori, origin of replication; RBCD, Reis–Bucklers corneal dystrophy; Re, reverse.

DNA sequencer (ABI Prism 377, Applied Biosystems, Foster, California, USA).

Microarray fabrication

The 3′ termini of designed probes were modified with amine residues using an amino-linker column (Cruachem, Glasgow, Scotland) for their immobilisation on the aldehyde-coated slide glasses (CEL Associates, Huston, Texas, USA) by forming amine–aldehyde covalent bonds. The 15mer-poly A oligonucleotide labelled with fluorescent dyes such as Cy3, Cy5 or fluorescein isothiocyanate (FITC) was used as position marker. The 11mer, 13mer, 15mer and 17mer probes were designed and tested for their efficiencies in detecting the mutations. The probes were resuspended in 3× saline sodium citrate spotting solution (0.45 M NaCl, 15 mM $C_6H_5Na_3O_7$, pH 7.0) and spotted on the slide glass using MicroGridII (BioRobotics, Cambridge, UK). After spotting, the slide glasses were air dried at room temperature for 12 h or baked at 80°C for 1 h.

Table 2 Probes designed for detecting the mutations in the βigh3 gene

n*	Mutations	Probe name	Length	Sequences (5′–3′)†	Exon number	Disease
1	R124	Not ACD (4-CGC)	11	GGACCGCACGG	4	Normal/heterozygotic
			13	CGGACCGCACGGA		
			15	**ACGGACCGCACGGAG**		
			17	CACGGACCGCACGGAGA		
2	R124	Not LCD I (4-CGC)	11	CGGACCGCACG	4	Normal/heterozygotic
			13	ACGGACCGCACGG		
			15	**CACGGACCGCACGGA**		
			17	ACACGGACCGCACGGAG		
3	R124H	ACD (4-CAC)	11	GGACCACACGG	4	ACD
			13	CGGACCACACGGA		
			15	**ACGGACCACACGGAG**		
			17	CACGGACCACACGGAGA		
4	R124C	LCD I (4-TGC)	11	CGGACTGCACG	4	LCD I
			13	ACGGACTGCACGG		
			15	**CACGGACTGCACGGA**		
			17	ACA CGG ACT GCA CGG AG		
5	R124L	RBCD (4-CTC)	11	GGACCTCACGG	4	RBCD
			13	CGGACCTCACGGA		
			15	**ACGGACCTCACGGAG**		
			17	CACGGACCTCACGGAGA		
6	R555	Not GCD (12-CGG)	15	AAGAGAACGGAGCAG	12	Normal/heterozygotic
			17	**CAAGAGAACGGAGCAGA**		
			16	CAAGAGAACGGAGCAG		
			16	AAGAGAACGGAGCAGA		
7	R555W	GCD (12-CGG)	17	**CAAGAGAATGGAGCAGA**	12	GCD
			16	CAAGAGAATGGAGCAG		
			16	AAGAGAATGGAGCAGA		

ACD, Avellino corneal dystrophy; GCD, granular corneal dystrophy; LCD I, lattice corneal dystrophy type I; RBCD, Reis–Bucklers corneal dystrophy.
*These probe numbers are used in fig 3A and fig 4A when showing the probe positions.
†The sequences of the finally selected probes used in DNA chips are shown in bold.

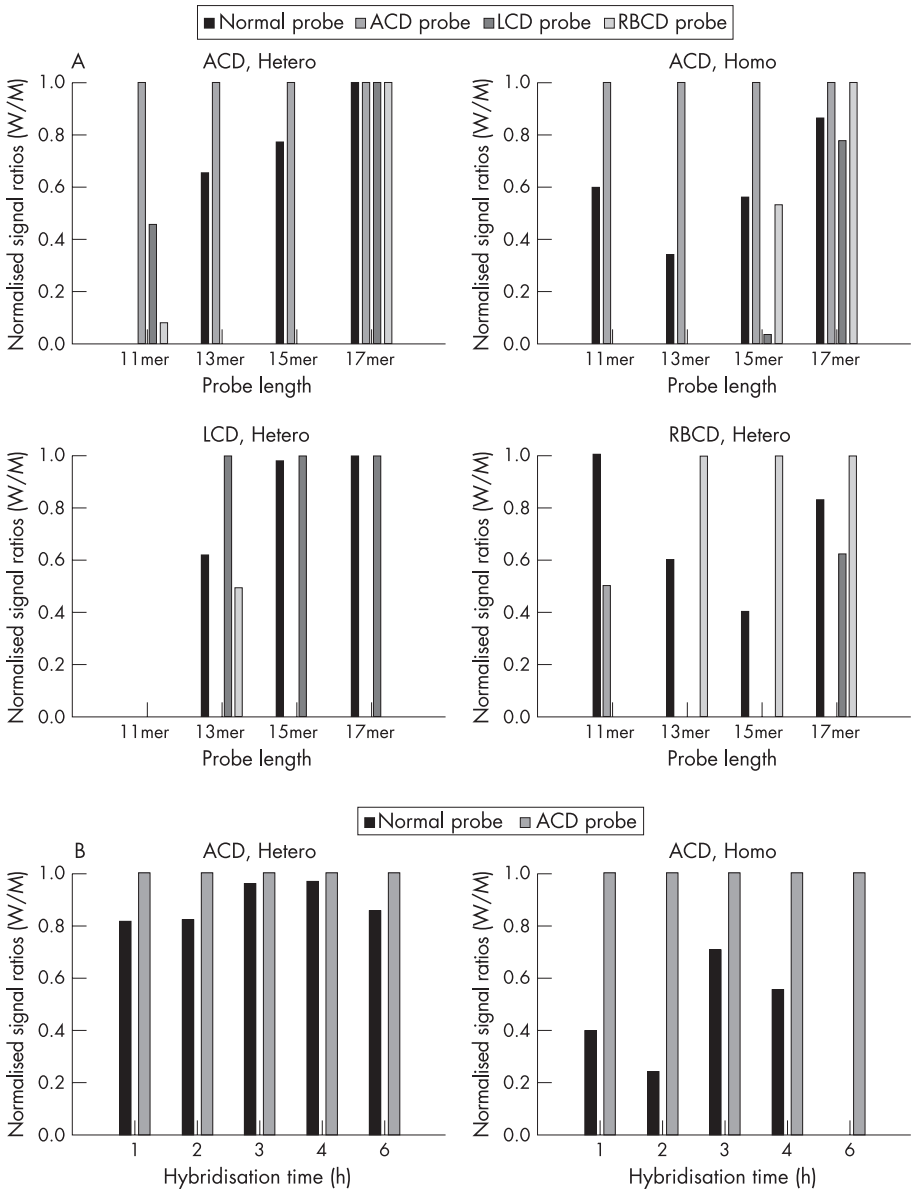

Figure 2 Hybridisation results according to the probe length (A) and the hybridisation time (B). The results are normalised for the signal obtained with the wild-type sequence versus that obtained with the mutant sequence (W/M). ACD, Avellino corneal dystrophy; LCD, lattice corneal dystrophy; RBCD, Reis–Bucklers corneal dystrophy.

Table 3 βigh3 gene mutations in 98 participants

Mutations	Sequences	Exon number	Number of cases	Disease
R124H	CGC to CAC			
	Heterozygotic	4	57	ACD
	Homozygotic		10	ACD
R124C	CGC to TGC			
	Heterozygotic	4	1	LCD I
R124L	CGC to CTC			
	Heterozygotic	4	1	RBCD
R555W	CGG to TGG	12	2	GCD
None	No mutation	4 and 12	27	
Total			98	

ACD, Avellino corneal dystrophy; GCD, granular corneal dystrophy; LCD I, lattice corneal dystrophy type I; RBCD, Reis–Bucklers corneal dystrophy.

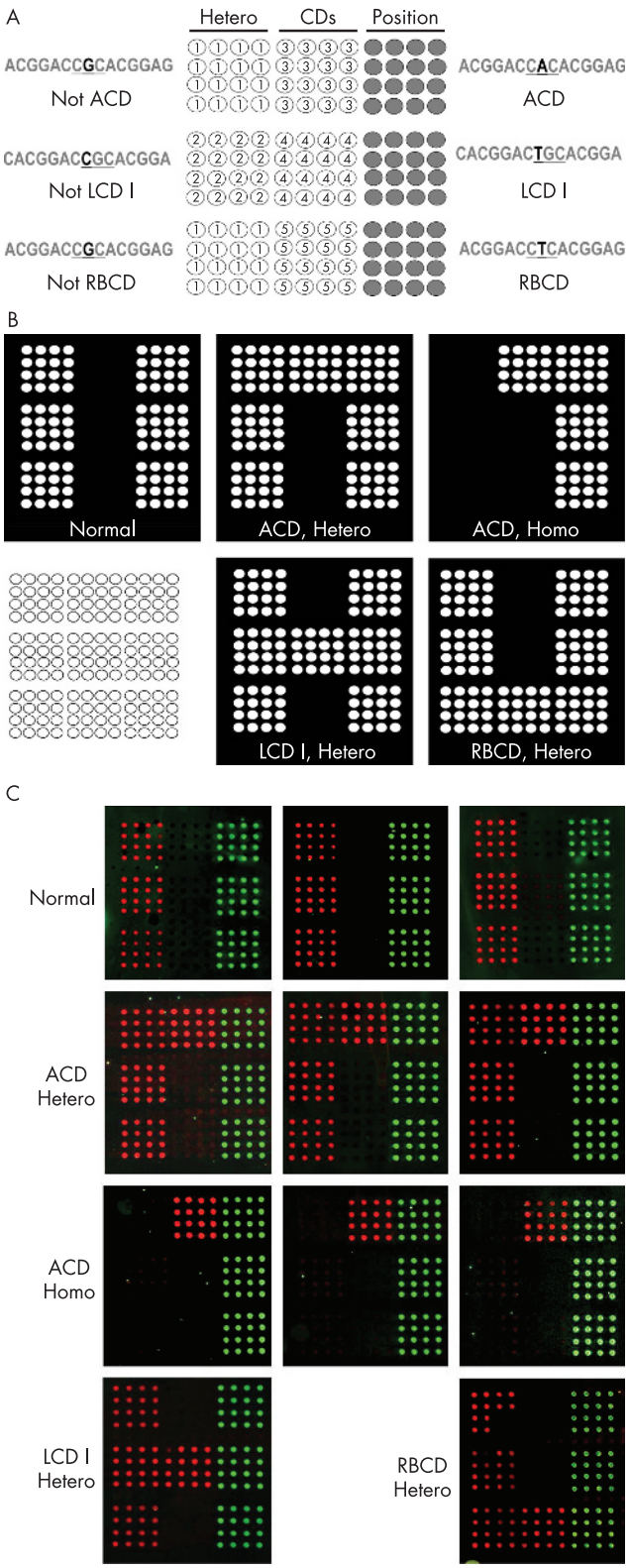

Figure 3 The first version of DNA chip designed for the detection of mutations in the βigh3 gene. (A) The DNA chip design and probe information. (B) Expected hybridisation results according to the types of the corneal dystrophies (CDs). (C) Representative DNA chip hybridisation results obtained using clinical samples. Position means the spotted position markers always showing fluorescence to tell the spot positions accurately. ACD, Avellino corneal dystrophy; LCD I, lattice corneal dystrophy type I; RBCD, Reis–Bucklers corneal dystrophy.

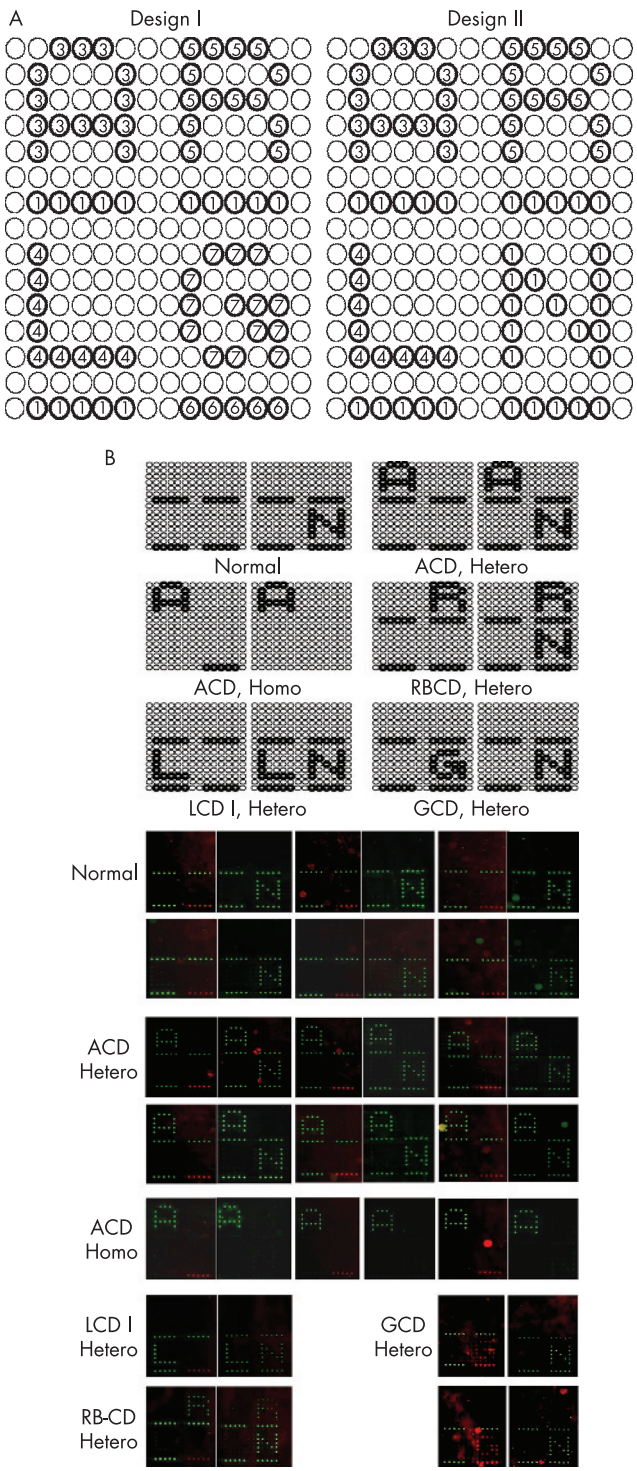

Figure 4 The final version of DNA chip designed for the detection of mutations in the βigh3 gene. (A) The DNA chip design using the probes listed in table 2. (B) Expected hybridisation results according to the types of corneal dystrophies, and the representative DNA chip hybridisation results obtained using clinical samples. ACD, Avellino corneal dystrophy; GCD, granular corneal dystrophy; LCD I, lattice corneal dystrophy type I; RBCD, Reis–Bucklers corneal dystrophy.

Table 4 Summary of DNA microarray-based detection of the βigh3 gene in this study

Type	Mutation	DNA microarray result		Sensitivity (%)*	Specificity (%)†	Positive predictive value (%)‡
		Positive	Negative			
Normal		27	0	100	100	100
Non-normal		0	71			
ACD						
Heterozygotic	CGC/CAC	57	0	100	100	100
Non-heterozygotic		0	41			
Homozygotic	CAC	10	0	100	100	100
Non-homozygotic		0	88			
LCD I						
Heterozygotic	CGC/TGC	1	0	100	100	100
Non-heterozygotic		0	97			
RBCD						
Heterozygotic	CGC/CTC	1	0	100	100	100
Non-heterozygotic		0	97			
GCD						
Heterozygotic	CGG/TGG	2	0	100	100	100
Non-heterozygotic		0	96			

ACD, Avellino corneal dystrophy; GCD, granular corneal dystrophy; LCD I, lattice corneal dystrophy type I; RBCD, Reis–Bucklers corneal dystrophy.
*Sensitivity is defined as the number of true positives divided by the sum of true positives and false negatives.
†Specificity is defined as the number of true negatives divided by the sum of true negatives and false positives.
‡Positive predictive value is defined as the number of true positives divided by the sum of true positives and false positives.

Preparation of target DNA

To prepare the target DNA, asymmetric PCR was carried out in a 50 µl reaction mixture containing 1× buffer, 0.2 mM deoxynucleotides triphosphates, 1 U Taq polymerase (Takara Shuzo, Shiga, Japan), 100 ng chromosomal DNA, 3 µM forward primer and 30 µM reverse primer. The 5′ terminus of each reverse primer used in asymmetric PCR was modified with Cy5, Cy3 or FITC. The ACD and Ori 12 primer sets were used for amplifying exons 4 and 12, respectively (table 1). By using a 10 times higher concentration of fluorescent-labelled reverse primer, mainly single-stranded DNAs which are antisense to the probes could be prepared. The ACD and Ori 12 primer sets were used for amplifying exons 4 and 12, respectively (table 1). The sizes of the amplified products from exons 4 and 12 were 147 and 318 bp, respectively(fig 1).

Hybridisation and data analysis

The spotted slides were steamed with boiling water for 1 min and soaked in 70% (v/v) ice-cold ethanol to remove any non-covalently bound oligonucleotides. To block the background fluorescence, the unreacted aldehyde groups were treated with sodium borohydride ($NaBH_4$) solution. The PCR-amplified target products (10 µl each) were directly added to the hybridisation solution containing 6× saline/sodium phosphate/EDTA (SSPE) (0.9 M NaCl, 10 mM $NaH_2PO_4 \cdot H_2O$, 1 mM EDTA, pH 7.4) and 20% (v/v) formamide. Hybridisation was carried out by incubating at 30℃ for 4–6 h in Array Chamber X (GenomicTree, Daejeon, Korea). After washing with 3× SSPE for 5 min, 2× SSPE for 5 min and 1× SSPE for 5 min, the slides were scanned using arrayWoRx (Applied Precision LLC, Issaquah, Washington, USA). Data were analysed by ImaGene 6.0 software (BioDiscovery, El Segundo, California, USA). The signal intensities were calculated by global normalisation for all positive spots, after subtracting the background values from the median signal intensities measured at 595 nm for Cy3, 685 nm for Cy5 and 530 nm for FITC.

RESULTS

The specific probes were designed on the basis of the sequences of the normal and mutant βigh3 genes (fig 1 and table 2). First, the probe length and the hybridisation time were optimised for the diagnosis of CDs. It was concluded that the use of 15mer probes and hybridisation at 30℃ for 4–6 h resulted in the highest sensitivity and specificity for detecting the mutations in exon 4 (fig 2). The wild type-to-mutant signal ratio, defined as the ratio of the spot signal of the wild-type probe for normal sequence (denoted as W) to that of each type of CD probe for each mutated sequence (denoted as M), was used for the probe optimisation. Each probe signal for the mutated sequence was set to one, and the ratios of other probe signals to each mutated sequence were calculated according to the probe length and the hybridisation time. Figure 2A shows that single-base mismatch could be discriminated successfully using a 13mer probe in the homozygotic ACD, but the 15mer probes showed the highest discrimination efficiency in different types of CDs. The discrimination can be further enhanced by changing the probe concentration. Hybridisation time was optimised with the heterozygotic ACD and the homozygotic ACD samples. As shown in fig 2B, hybridisation for 4–6 h was found to be optimal for the highest sensitivity and specificity. The probes finally selected are shown in bold letters in table 2.

The DNA chip-based diagnosis of CDs was performed using blind tests for a total of 98 blood samples. The genotypes of these samples were also determined by direct sequencing for the verification of DNA chip results. DNA sequence analysis showed that the 98 participants could be classified as 27 normal, 10 homozygotic ACD, 57 heterozygotic ACD, 1 heterozygotic LCD I, 1 heterozygotic RBCD and 2 GCD (table 3).

All microarray experiments were carried out in duplicate. The results of DNA chip-based diagnosis matched 100% with those obtained with direct DNA sequencing for all 98 samples. Four types of CDs—namely, homozygotic ACD, heterozygotic ACD, heterozygotic LCD I and heterozygotic RBCD—were detected in 96 samples. Figure 3 illustrates the DNA chip design, the expected results and the representative DNA chip hybridisation results. We also designed the probes for detecting GCD (two samples) in an attempt to analyse the possibility of detecting other types of CDs with DNA chip technology. Figure 4 shows the applied DNA chip design and the representative results. The DNA chip developed in this study could successfully diagnose different types of CDs with 100% sensitivity, 100% specificity and 100% positive predictive value. Table 4 summarises the DNA chip hybridisation results of blind samples. It should be noted that ACD is the most common type of CD detected in this study. Even though we cannot truly claim the high specificity, sensitivity and the predictive value for other types of CDs as the number of samples was too small, the results clearly showed

that the DNA microarray developed in this study can successfully distinguish between different types of CDs.

DISCUSSION

CDs have most often been categorised by their clinical appearance examined by light and electron microscopes. However, their appearance is variable, and thus is often misdiagnosed. Recently, various types of mutations causing different CDs have been reported,[8–11] and the molecular basis for the common and rare CDs is becoming apparent. However, the disease phenotypes of CDs seem to be more diverse than expected previously. Thus, it is desirable to diagnose them accurately by direct DNA sequencing and related genetic analyses. In this paper, we reported the development of a DNA chip that allows simple, yet highly specific and accurate, detection of mutations in the βigh3 gene causing the CDs. The discrimination of the single-base mismatch from the perfect match could be optimised by examining the different lengths and concentrations of probes and the hybridisation time. Through the blind tests, we were able to conclude that this DNA chip-based method allows successful detection of various mutation types of the CDs in 98 samples. Thus, the DNA chip developed in this study allows specific and accurate diagnosis of multiple types of β-aCDs, which should be useful in the present clinical diagnosis and classification of β-aCDs.

ACKNOWLEDGEMENTS

This work was supported by Medigenes. Further support by the LG Chem Chair Professorship (SYL) is appreciated.

Authors' affiliations

So Young Yoo, Department of Chemical and Biomolecular Engineering (BK21 program), Korea Advanced Institute of Science and Technology, Daejeon, Korea; Medigenes, Seoul, Korea
Tae-Im Kim, Eung Kweon Kim, Corneal Dystrophy Research Institute, Department of Ophthalmology, Project Team of Nanobiomaterials for Cell-based Implants, Severance Hospital, Yonsei University, Seoul, Korea
Sang Yup Lee, Department of Chemical and Biomolecular Engineering (BK21 program), Centre for Systems and Synthetic Biotechnology, Institute for the BioCentury, Korea Advanced Institute of Science and Technology, Daejon, Korea
Ki Chang Keum, Department of Radiation Oncology, Yonsei University College of Medicine, Seoul, Korea
Nae Choon Yoo, Department of Oncology and Cancer Centre, Yonsei University College of Medicine, Seoul, Korea
Won Min Yoo, Department of Plastic and Reconstructive Surgery, Yongdong Severance Hospital, Seoul, Korea

Competing interests: None.

Correspondence to: Professor S Y Lee, Department of Chemical & Biochemical Engineering, Korea Advanced Institute of Science and Technology (KAIST), 373–1 Guseong-dong, Yuseong-gu, Daejeon 305–701, Korea; leesy@kaist.ac.kr; Professor E K Kim, Department of Ophthalmology, Yousei University College of Medicine, Seodaemoongu Shinchondong 134, C PO Box 8044, Seoul, Korea; eungkkim@yumc.yonsei.ac.kr

Accepted 1 January 2007
Published Online First 10 January 2007

REFERENCES

1 **Afshari NA**, Mullally JE, Afshari MA, *et al*. Survey of patients with granular, lattice, avellino, and Reis-Bucklers corneal dystrophies for mutations in the BIGH3 and gelsolin genes. *Arch Ophthalmol* 2001;**119**:16–22.
2 **Chau HM**, Ha NT, Cung LX, *et al*. H626R and R124C mutations of the TGFBI (BIGH3) gene caused lattice corneal dystrophy in Vietnmese people. *Br J Ophthalmol* 2003;**87**:686–9.
3 **Ellies P**, Renard G, Valleix S, *et al*. Clinical outcome of eight BIGH3-linked corneal dystrophies. *Ophthalmology* 2002;**109**:793–7.
4 **El-Ashry MF**, Abd El-Aziz MM, Ficker LA, *et al*. BIGH3 mutation in a Bangladeshi family with a variable phenotype of LCDI. *Eye* 2004;**18**:723–8.
5 **Konishi M**, Yamada M, Nakamura Y, *et al*. Varied appearance of cornea of patients with corneal dystrophy associated with R124H mutation in the BIGH3 gene. *Cornea* 1999;**18**:424–9.
6 **Yoshida S**, Yoshida A, Nakao S, *et al*. Lattice corneal dystrophy type I without typical lattice lines: role of mutational analysis. *Am J Ophthalmol* 2004;**137**:586–8.
7 **Mitterer G**, Huber M, Leidinger E, *et al*. Microarray-based identification of bacteria in clinical samples by solid-phase PCR amplification of 23S ribosomal DNA sequences. *J Clin Microbiol* 2004;**42**:1048–57.
8 **Aldave AF**, Gutmark JG, Yellore VS, *et al*. Lattice corneal dystrophy associated with the Ala546Asp and Pro551Gln missense changes in the TGFBI gene. *Am J Ophthalmol* 2004;**138**:772–81.
9 **Hirano K**, Hotta Y, Fujiki K, *et al*. Corneal amyloidosis caused by Leu518Pro mutation of betaig-h3 gene. *Br J Ophthalmol* 2000;**84**:583–5.
10 **Hirano K**, Hotta Y, Nakamura M, *et al*. Late-onset form of lattice corneal dystrophy caused by leu527Arg mutation of the TGFBI gene. *Cornea* 2001;**20**:525–9.
11 **Warren JF**, Abbott RL, Yoon MK, *et al*. A new mutation (Leu569Arg) within exon 13 of the TGFBI (BIGH3) gene causes lattice corneal dystrophy type I. *Am J Ophthalmol* 2003;**136**:872–8.

Development of a DNA chip for the diagnosis of the most common corneal dystrophies caused by mutations in the βigh3 gene

So Young Yoo, Tae-Im Kim, Sang Yup Lee, et al.

Br J Ophthalmol 2007 91: 722-727 originally published online January 10, 2007
doi: 10.1136/bjo.2006.111070